APC-targeted DNA vaccines against pandemic influenza

Doctoral thesis by

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On a personal note. Life exists of many phases. Each of the phases are defined by certain elements, such as the work or projects that were carried out at the time, or the people that were important to you. As I now present this work, it represents the end of a phase, the phase as a doctoral research fellow. I imagine that I will think of this phase as a time where I matured as a scientist and life comfortably continued to settle in. Many people will remain strong in the memories of these days.

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### General Discussion
Introduction

The key concept of prophylactic vaccination is to induce immunological memory so that upon exposure, a pathogen can be quickly recognized and eliminated. An immune individual will thus not experience the same infectious disease twice. Voltaire described this principle in his writings in "Philosophical Letters" from 1742 where he discusses inoculation and how the Circassian people have discovered and practice immunization:

"The Circassians found that, upon computation, in a thousand persons there was hardly one that was ever twice seized with smallpox completely formed; that there had been instances of a person's having had a slight touch of it, or something resembling it, but there never were any two relapses known to be dangerous; in short, that the same person has never been known to have been twice infected with this disorder."

Voltaire, Philosophical Letters XI - Inoculation, 1742 [1].

Voltaire writes about how the Turks have derived this method from Circassia, and reports that the Chinese have practiced similar immunization for hundreds of years. More recent data indicate that the practice had been performed in the Song Dynasty in China already during the 10th century [2].

Today we acknowledge the incredible success story of vaccination in modern medicine. Vaccinations have essentially eradicated a variety of infectious diseases such as smallpox, polio, diphtheria, measles, and more [3]. However, some infectious diseases are more or less impossible to eradicate due to evasion strategies that have evolved [4]. One of these diseases is influenza, caused by the influenza virus.

Influenza viruses are found in several species of animals, including birds, bats, pigs and humans. Seasonal influenza is caused by virus circulating in the human population. Influenza is easily transmittable. Rapid mutations in the antigenic structure mediate immunological escape and allow the virus to be continuously present in the global population. Although the rapid mutations result in sub-optimal vaccine efficacy, World Health Organization (WHO) and Centers for Disease Control and Prevention (CDC) surveillance programs allow seasonal influenza vaccines to be produced in advance of the seasonal outbreak. These vaccines can prevent spread and disease.

More extensive mutations or recombination of different influenza viruses from human or other species can form novel viruses to which the human population is immunologically naïve. Such influenza viruses can cause a pandemic outbreak [5]. There is no method
to predict with certainty the next virus that will cause a pandemic outbreak as was demonstrated during the most recent pandemic outbreak in 2009 [6]. Few, if any, expected an H1N1 serotype to cause the next influenza pandemic, which resulted from a triple reassortment of genes from avian, swine and human origins [7, 8]. Today, surveillance indicates that a highly pathogenic avian influenza virus (HPAIV) of an H5 or H7 subtype is more likely to cause the next pandemic outbreak [9–11]. However, the production of a pandemic influenza vaccine cannot start before the pandemic influenza virus has been identified, and current manufacturing strategies are insufficient in the face of a pandemic outbreak.

To overcome these challenges, new and more effective vaccines are necessary. We need safe vaccines that can be quickly manufactured, adapted, and administered in the face of a pandemic threat. In this thesis, strategies and investigations into the development of such vaccines are presented. A vaccine candidate against HPAIV H7 is presented. The vaccine targets hemagglutinin (HA) to antigen presenting cells (APCs) to increase immunogenicity and accelerate the immune response. The underlying mechanism behind the enhanced immune response elicited by APC targeted vaccines is investigated.

The Immune System

The immune system is a complex network made up by a plethora of cells in multiple organs spanning the entire body. Entities of the immune system perform unique tasks working together to form a multifaceted defense against foreign material. In order to attack and clear invading pathogens in a safe and specific manner, the immune system needs to discriminate between self and non-self. Additionally, the immune system needs to be able to establish an appropriate immune response, and then regulate and terminate the response. This is important so that there will always be space in lymphoid organs for fighting new pathogens and that the damage inflicted by inflammation is minimized. The immune system is a finely tuned and complex system continuously balancing immunological combat with self preservation.

The Innate Immune System

The innate immune system is inherited and forms the first line of defense against foreign material. In addition to physiological barriers such as the skin and mucous membranes, agents of the innate immune system quickly recognize and eliminate invading microor-
organisms. The multiple effectors of the innate immune system utilize germline encoded mechanisms that have evolved through natural selection, and include phagocytic cells, natural killer cells and a huge number of various interacting proteins such as complement and small antibacterial peptides known as defensins [12, 13]. In order to discriminate between self and non self, the immune system use pattern recognizing receptors (PRRs) [14]. These receptors recognize pathogen associated molecular patterns (PAMPs) such as double stranded viral RNA, unmethylated CpG motifs in DNA, sugar moieties such as bacterial lipopolysaccharides and endotoxins, as well as the absence of markers of normal self such as sialic acid residues that normally cover mammalian cells [15]. These cues trigger the innate immune system and the vast majority of invading pathogens are cleared within minutes by agents of the innate immune system. However, the innate immune system is not specific and does not acquire immunological memory, which are the strengths of the adaptive immune system.

The Adaptive Immune System

Immunological memory is formed, or adapted, by exposure to foreign pathogens and acquired throughout the lifetime of an individual. This adaptation and memory lay the foundation for vaccination. The immune system remembers the first encounter and can quickly eliminate pathogens upon a second exposure and thus avoid disease.

Cells of the adaptive immune system can respond specifically to virtually any invading pathogen and mount an appropriate response. As the naïve cellular repertoire of the adaptive immune system literally contains millions of receptors of various specificities, a specific encounter with an invading pathogen seems at first glance unlikely. To enhance the chance of a proper antigen encounter and the possibility for rapid initiation of the response, antigen encounter happens at specific sites in secondary lymphoid tissue such as the spleen and lymph nodes (LNs) (Fig.1). These organs function as molecular sieves where lymph and interstitial fluid is filtered through the LNs and blood is filtered through the spleen. LNs are strategically positioned at branch points in the lymphatic system, enabling efficient screening of antigens [16]. In fact, antigen can be detected in draining LNs within minutes of subcutaneous administration [17], and lymphocyte egress from LNs is then reduced [18] to increase the chance of antigen encounter. Furthermore, secondary lymphoid organs are internally organized with a specialized micro-architecture that optimizes antigen encounter, cellular interactions, and initiation of an immune response.

The LN is divided into three distinct regions based on the local chemokine and cellular
milieu (Fig. 1). The medulla contains B- and T cells, as well as macrophages and plasma cells. The paracortex is constituted by T cells, while B cells and follicular dendritic cells (DCs) settle in the follicles of the cortex [19]. Antigen-laden lymph enters the LN via the afferent lymph into the subcapsular sinus, through to the trabecular sinuses, and into the medulla before exiting through the efferent lymph vessel. Lymphatic fluid also flows through the conduit network, a collagen rich fibrous network allowing small molecules, such as small antigens and chemokines, to enter the paracortex through high endothelial venules (HEVs). HEV also connects the LNs with the blood stream, creating a site specific area for entry and exit of lymphocytes while forming a seal between lymphatic tissue, potentially containing pathogens, and the blood stream.

The spleen is structurally similar to the LNs, with a conduit system, T cell zones and B cell follicles [19]. These compartments are localized in the white pulp in the spleen, surrounded by red pulp, separated by the marginal zone containing high amounts of macrophages, DCs, and B cells [20]. The spleen is tightly connected to the blood stream through vascular rich red pulp. Extensive vasculature sends blood from central arteriole branches in the marginal sinus through the marginal zone into the red pulp. In contrast to LNs, the spleen is not connected with lymphatic fluid and is specialized in mounting an immune response against blood-borne antigens [21].

Figure 1: Schematic illustration of the anatomy of secondary lymphoid organs. The LN is dived into three internal regions based on local chemokine and cellular milieu; the medulla, the paracortex, and the follicles. In the spleen the white pulp consists of the paracortex, B cell follicles, central arteriole and the marginal zone. Adapted from Batista et al. [19]
**B cells**

The main purpose of B cells is to produce immunoglobulins (Ig), or antibodies, that can specifically attach to antigens and neutralize them. B lymphocytes originate from hematopoietic stem cells in the bone marrow in mammals and migrate to secondary lymphoid organs such as the LNs or spleen where they settle in the follicles (Fig. 1) [19]. The mechanisms involved in transporting antigen to B cells and DCs in the follicles is not fully understood. Small molecules of less than approximately 70kDa can diffuse directly into the paracortex through HEV [22]. Larger molecules do not travel freely over the LN parenchyma [23], but are actively transported by subcapsular sinus macrophages [24]. These macrophages span the capsule with a head protruding into the subcapsular sinus and a long tail extending into the follicle, sampling and transporting material into the follicles [25]. Subcapsular sinus macrophages act as gatekeepers at the lymph-tissue interface, allowing efficient encounter of antigen without compromising LN integrity or risking systemic spread of an invading pathogen [26]. In contrast to other macrophages, subcapsular sinus macrophages exhibit limited phagocytic activity and facilitate intact antigen presentation to B cells in the follicles [27]. Thus, antigen can be presented directly to B cells, or loaded on follicular DCs that present antigen to B cells for an extended period of time [28]. These processes are especially efficient if the antigen is able to form immune complexes [29, 30]. Furthermore, B cells that enter the LNs and migrate across the T cell area can be activated upon encounter with antigen loaded on DCs. In fact, recently immigrated B cells concentrate around HEV in the paracortex, forming contact with DCs before entering the follicles [31].

B cells are activated by engagement of the B-cell receptor (BCR), a transmembrane receptor protein composed of a membrane bound antibody. Self reactive B cells are clonally deleted or rendered anergic in the bone marrow or periphery [32, 33]. Upon ligand induced activation, the BCR forms a complex with the signal transduction moiety Ig-α and Ig-β heterodimer, known as CD79. These membrane bound proteins have a cytoplasmic tail bearing the immunoreceptor tyrosine-based activation motif (ITAM). Activation of the BCR leads to phosphorylation of the ITAMs and induction of downstream activation pathways [34]. BCRs are effectively activated upon engagement with membrane bound antigens, and form a supramolecular activation cluster (SMAC) that is part of an immunological synapse (Fig. 2) [35]. This synapse formation ensures efficient B cell activation and can lower the threshold of antigen necessary for B cell activation [36, 37].

Upon proper engagement of the BCR, B cells will phagocytose the antigen, process it and
present it to T cells via major histocompatibility complex (MHC) class II molecules. In return, T cells recognizing the antigen-MHCII complex will supply help to the B cell, and interact with co-stimulatory receptors and cytokines to stimulate further B cell development, class switch recombination, and affinity maturation through somatic hypermutations (SHM) [39, 40]. This will result in B cell populations with high affinity antibodies that can differentiate into long lived memory or plasma cells [41]. Plasma cells migrate to the bone marrow and secrete antibodies for extended periods of time. They can persist in the bone marrow for several decades or even the entire lifetime of an individual [42, 43]. Memory B cells and plasma cells express high amounts of MHCII and have high affinity for the antigen. Upon a second encounter with the antigen, memory B cells will be rapidly activated and high affinity antibodies will be quickly produced.

Immunoglobulin Structure and Function

Igs, or antibodies are large glycoproteins consisting of two identical heavy and light chains (Fig.3). These chains are held together by non-covalent bonds and disulfide bonds. The chains consist of opposing β-sheets forming Ig domains. The light chains consist of two Ig domains, one variable (V<sub>L</sub>) and one constant (C<sub>L</sub>). Likewise, the heavy chains consist of 3-4 constant domains (C<sub>H1</sub> - 3/4) and one variable domain (V<sub>H</sub>). In the V<sub>L</sub> and V<sub>H</sub> domains, complementarity-determining regions (CDRs) are formed by three loops named CDR1, CDR2, and CDR3 which form the antibody binding site (paratope) [44]. The light chains and the upper half of the heavy chains form the arms on the classical Y-shape of monomeric Ig referred to as the Fab-region, where the antigen binding sites are localized. The lower half of the heavy chain segments form the Fc region of an Ig, and have a role in modulating various effector functions by Fc receptor (FcR) binding and complement activation [45].
Igs must recognize a virtually unlimited number of unknown antigens. To achieve this level of diversity from a finite number of germline gene sequences, genes in the Ig locus form complete heavy and light chain exons by V(D)J recombination, a process mediated by recombination-activating genes (RAGs) [46, 47]. The Ig heavy chain is generated by somatic recombination of variable (V), diversity (D) and joining (J) gene segments, and the light chain by recombination of V and J segments. By these mechanisms the preimmune antibody repertoire likely consists of more than $10^{12}$ different antibody clones generated from the Ig locus. Secondary diversification such as isotype switching and SHM mediated by activation-induced cytidine deaminase (AID) in the germinal center reaction lead to further diversification of the antibody repertoire.

![Diagram](image)

**Figure 3:** Schematic illustration of an IgG antibody and variable light and heavy domains. A) An IgG antibody consisting of identical heavy (orange) and light (blue) chains held together by disulfide bonds. A hinge region connects the Fab and Fc fragments ensuring flexibility for the antigen binding site. Small dots in the CH2 domain indicate glycosylation sites. B) Ig variable region is composed of an Ig-fold with nine antiparallel $\beta$ strands folded into two $\beta$ sheets. Adapted from Lunde et al. [48]

Light chains can be either $\lambda$ or $\kappa$ chains, while there are five isotypes, $\alpha$, $\delta$, $\epsilon$, $\gamma$, and $\mu$, of heavy chains that will determine the effector functions of Igs. A naïve B cell expresses IgD and IgM BCRs. Although BCRs have the potential to recognize virtually unlimited amounts of antigens, a BCR is likely to bind many antigens with a fairly low affinity. To increase ligation of the BCR by antigen, the IgM receptor is pentameric, thus increasing its avidity and likelihood of proper ligation upon encounter with antigen. Activated B cells secrete pentameric IgM as an early defense mechanism and then switch heavy chains
to secrete IgA, IgG or IgE antibodies as plasma cells. A single B cell progenitor can be clonally expanded to produce various Ig isotypes of identical specificity. IgA antibodies are secreted as dimers called polymeric IgA which are actively transported across epithelia in mucosal barriers by the polymeric IgA receptor [49]. Thus IgA is prevalent in secreted fluids at mucosal sites and primary exerts its effect by neutralizing invading pathogens at epithelial borders [50]. IgG is the most abundant isotype in blood and interstitial fluid and constitutes approximately 75% of serum antibodies. IgG antibodies are further divided into four subclasses; IgG1, IgG2, IgG3, and IgG4 in humans and IgG1, IgG2a, IgG2b, and IgG3 in mice [39,51]. These isotypes of IgG have various effector functions but are generally efficient in neutralizing pathogens, complement activation and opsonizing pathogens. Variations in effector functions for the different IgG subclasses are mediated by their affinity for various FcRs, as the affinity ratios for inhibitory or activating FcRs predict activity [52]. IgE antibodies are the least abundant in serum, and have been shown to be involved in triggering inflammatory responses to parasitic infections and allergic reactions [53].

T cells

While the primary task of B cells is to produce antibodies and establish humoral immunity, T cells mediate cellular immunity. The main task for T cells is to provide help to B cells, or become cytotoxic T cells that kill infected cells. Similar to B cells, T cells originate in the bone marrow from hematopoietic stem cells, but progenitor cells migrate to the thymus before entering the periphery.

In the thymic cortex, progenitor T cells develop into double positive T cells (CD4+ CD8+) and later single positive (CD4+ or CD8+) [54]. When here, T cells that bind peptide-MHC complexes weakly, receive a survival signal, and T cells that bind peptide-MHC complexes too strongly are deleted. This is called positive and negative selection, respectively [55]. Thus, T cells are ensured to recognize foreign antigenic peptides presented on MHC in the periphery, but not react to self antigens. T cells develop into CD8 or CD4 single positives upon transition to the medulla, depending on whether the T-cell receptor (TCR) recognizes antigen presented on MHCI or MHCII, respectively.

T cells are divided into two main groups; CD8+ cytotoxic T-lymphocytes (CTL) and CD4+ T-helper cells. CD8+ CTL are central in clearing cells infected with intracellular pathogens. CTL can directly kill infected cells by two specific pathways; perforin and granzymes, or Fas-Fas ligand interactions. Upon encounter with an infected cell, CTL
secretes perforin that oligomerizes to form pores in the target cells, breaking cell integrity and mediating influx of granzymes [56]. Simultaneously, CTL can induce cell death by Fas-Fas ligand interactions [57, 58]. Both of these pathways lead to DNA fragmentation and apoptosis of the target cell that is then cleared by adjacent macrophages.

CD4+ T helper cells are further divided into; Th1, Th2, Th17, follicular T helper cells (T\textsubscript{FH}), and regulatory T cells (Treg) [59]. These functionally distinct subsets confer immune responses mediating proper host defense and immune regulation. Signature cytokines and master regulators determine the fate of a developing T cell, as indicated in Table 1. However, helper T cell subsets exhibit flexibility and plasticity in their responses, and single CD4+ T cells have been observed to express more than one signature cytokine in vivo [60, 61]. T helper cell plasticity allows for adaptation between regulatory and cytokine-producing effector cells, as well as modulation of the cytokine profile during an immune response.

Th1 cells provide help to cytotoxic cells such as macrophages and CD8+ CTL while Th2 cells help establish humoral immunity. T\textsubscript{FH} trigger and maintain germinal centers and are key in providing help to germinal center B cells through CD40/C40L interactions [62,63]. Th17 cells are important in maintaining tissue homeostasis and clearing pathogens at mucosal barriers. They are also involved in inflammatory responses and autoimmune disease [64]. Tregs are essential in dampening the immune responses. Mice lacking Tregs develop fatal autoimmune pathology indicated by Th1 and Th2 cytokine signatures [65].

<table>
<thead>
<tr>
<th>T helper Subset</th>
<th>Master Regulator</th>
<th>Signature Cytokines</th>
</tr>
</thead>
<tbody>
<tr>
<td>Th1</td>
<td>T-bet</td>
<td>IFN-\gamma, IL-12</td>
</tr>
<tr>
<td>Th2</td>
<td>Gata3</td>
<td>IL-4, IL-5, IL-13</td>
</tr>
<tr>
<td>Th17</td>
<td>ROR\gamma t</td>
<td>IL-17, IL-22</td>
</tr>
<tr>
<td>T\textsubscript{FH}</td>
<td>Bcl6</td>
<td>IL-4, IL-21</td>
</tr>
<tr>
<td>Treg</td>
<td>FoxP3</td>
<td>IL-10, TGF-\beta</td>
</tr>
</tbody>
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Table 1: T helper cell signature cytokines and master regulators [59,66].

**The T-cell Receptor**

Similarly to B cells and the BCR, T cells execute specificity based on their TCR. In contrast to the BCR, the TCR does not recognize complete antigen, but can bind peptide fragments of antigen presented on MHC. All nucleated cells of jawed vertebrates present intracellular peptides on MHC class I molecules. Recognition of foreign material
presented on MHC class I indicates an intracellular pathogen, and will lead to killing by CTL. The T helper cell subset can recognize peptides presented on MHC class II by APCs that sample their surroundings and encounter pathogens in the extracellular space.

The TCR is a heterodimer made of two membrane bound protein chains. Most of the T cells bear a TCR consisting of an α and a β chain, while lower numbers of T cells have a γ and δ chain, referred to as αβ or γδ T cells, respectively. αβ TCRs form a TCR complex where two extracellular chains (α and β) are made by a variable and a constant domain formed by antiparallel β sheets known as the Ig fold. These chains are linked by disulfide bonds and form a complete TCR complex with the CD3 signal transduction complex containing intracellular ITAMs [67]. αβ T cells recognize antigenic peptide presented on MHC while γδ T cells are not MHC restricted. They are instead involved in recognition of lipid antigens and heat shock proteins, and are considered to be a link between the innate and adaptive immune system [68].

The somatic variability of the TCR follows the same principles of V(D)J rearrangement as for the generation of BCRs. The TCR α chain is generated by VJ rearrangement while the β chain is generated by VDJ rearrangement of genes in the TCR α and β loci, respectively. Somatic rearrangements of gene segments in the Ig and TCR loci are mediated by the same enzymes, emphasizing the fundamental similarity between these two processes. Great diversity of TCRs are generated by hypervariable loops located at the end of the TCR which forms the binding site for the MHC-peptide complex. The hypervariable CDR3 loops are generated from the (D)J gene segments, and CDR1 and CDR2 peripheral loops are formed by the V segments [69]. This genetic rearrangement results in a naïve T cell repertoire that is estimated to be a few orders of magnitude higher than the germline encoded BCR repertoire, however T cells do not undergo SHM.

**Antigen Presenting Cells**

B- and T cells are mediators and effectors of adaptive immunity. APCs, and in particular DCs, initiate and control the immune response. Antigen presentation involves presenting pieces of antigen on MHC molecules, principally making all nucleated cells APCs. However, APCs usually refer to professional APCs that can activate a naïve T cell, such as B cells, macrophages and DCs. These cells present antigen on MHC class I and II, facilitating presentation to both MHC class I restricted CD8+ and MHC class II restricted CD4+ T cells. T cells that recognize antigenic peptide on MHC can then be activated depending on the co-stimulatory molecules displayed by the APC.
MHC class I and II molecules facilitate presentation of antigenic material originating from different sources. For MHCI, the goal is to report on antigenic material originating from intracellular sources, such as a viral infection, to CD8+ T cells. MHCII molecules present peptide derived from sampling the extracellular environment and present antigen to CD4+ T cells. However, these pathways are not strict as cross presenting DCs can present exogenous antigen on MHCI molecules [70]. This is an important line of defense because for malignant cells or pathogens that do not infect APCs, CTL can only be activated if APCs present extracellular peptide on MHCI. Additionally, endogenous peptides can be presented on MHC class II through delivery of material to endosomes via autophagy [71]. Importantly, these processes have a profound influence on the of nature the immune response.

MHC class I molecules consist of a larger membrane bound α heavy chain folded into three domains, α1, α2, and α3 that are associated with β2 microglobulin. The α1 and α2 domains form the peptide binding groove which holds peptides of 8-10 amino acids in length. These peptides are mostly derived from defective ribosomal translation products degraded by proteasomes in the cytosol. Peptides are loaded into the endoplasmic reticulum (ER) where MHC class I molecules are synthesized and loaded with peptide, before the MHC class I peptide complex is transported to the cell surface [72].

MHC class II molecules are heterodimers of membrane bound α and β chains where the tip form the peptide binding groove. When MHC class II molecules are formed in the ER, the complex is stabilized by the chaperone invariant chain for proper folding, trafficking, and protection of the peptide binding groove from loading peptides in the ER. MHC class II molecules are then transported to late endosomes referred to as MIIC (MHC class II compartments), or to the cell surface where they are recycled back to MIIC where the invariant chain is replaced by a peptide of 13-17 amino acids [73,74].

B cells, macrophages, and DCs are professional APCs. B cells are generally considered poor APCs since antigen uptake is restricted to antigen recognition by the BCR [75–77]. Macrophages have exceptional phagocytic capability. Macrophages engulf pathogenic material that can activate the cell through ligation of PRRs [78]. Macrophages are also important in the gatekeeping and antigen presentation at the lymph tissue interphase in secondary lymphoid organs [25]. DCs are particularly potent as APCs and play a key role in shaping the immune response. These cells function as sentinels probing the local environment by constantly sensing self and non-self antigens. They either activate or
tolerize T cells [79]. Upon TLR stimulation, DCs are activated and up-regulate MHC class II and other co-stimulatory molecules before migrating to secondary lymphoid organs where they interact with B and T cells.

**Influenza**

Influenza is an infectious disease caused by the influenza virus. Common symptoms are head- and muscle-ache, chills, fever, sore throat, fatigue and etc. The influenza virus is very common and wide spread, infecting 5-15% of the global population causing on average half a million deaths annually with a huge socioeconomic impact [80]. Seasonal influenza originates in East and South-East Asia where overlapping epidemic waves feed the spread of virus to temperate regions of the world [81].

Constant evolution of the antigenic structure of the influenza virus allows the virus to persist as a pathogenic agent in humans. Immunological escape mechanisms include antigenic drift and antigenic shift. Antigenic drift is small changes caused by point mutations affecting the antigenic structure. The low replication fidelity of RNA polymerases with poor proofreading abilities makes such mutations an inherent trait of the influenza virus. Furthermore, the reassortment of gene segments of replicating viruses is, at least, partly random. This enables antigenic shift through complete substitution of certain gene segments, resulting in a potentially novel combination of genes in cells that are infected with two different parent viruses [82]. Whereas genetic drift cause seasonal epidemics, antigenic shift can result in novel strains with pandemic potential.

The influenza virus is an orthomyxoviridae virus characterized by the negative sense single stranded RNA genome. Influenza viruses are divided into four subgroups; influenza A, B, C, and D based on antigenic differences in the nucleoprotein (NP) and matrix protein (M1) [83]. Influenza viruses A, B, and C can infect humans, where A can cause severe pandemics and B cause milder epidemics. Influenza virus C cause mild respiratory disease and rarely cause epidemics [84].

Influenza viruses infect many species of animals. Aquatic birds are the natural reservoir for all influenza A viruses and represent a primordial source of influenza for many other species [85]. The broad amphotropism of influenza A viruses constitutes a threat of disease and pandemic potential to humans. Influenza A viruses adapted to an avian reservoir have a very limited capacity to infect and replicate in humans, but swine are susceptible to infections from influenza from both human and avian origin and constitute
a weak zoonotic barrier. Swine thus function as a zoonotic mixing vessel generating novel virus reassortments [86]. Introduction of avian influenza viruses to swine with subsequent adaptation might be a predisposition for passage on to humans. Such viruses hold pandemic potential as the human population are likely to be serologically naïve to the reassorted virus. Influenza A viruses are classified based on their HA and neuraminidase (NA) subtypes. Almost all subtypes have been identified in birds, where viral combinations of 16 HA (H1-16) and 9 NA (N1-9) are found [4]. Recently two influenza-like viruses have been derived from bats (H17N10 and H18N11) [87, 88]. Among these influenza A serotypes, 8 HA (H1-3,H5-7, and H9-10) and 6 NA (N1-2, and N6-9) have been described in humans [83]. Influenza B and C viruses are found almost exclusively in humans, but have been identified in horses, swine and seals [89,90].

**Structure and Function of the Influenza A Virion**

The influenza A virus has eight RNA segments encoding at least 16 known gene products [91]. The RNA genome segments are packed in ribonucleoprotein complexes containing RNA polymerase (PB1, PB2, and PA) and NP. The internal parts of the virus are structured by the M1 matrix protein that mediates encapsidation by binding the viral membrane and RNA simultaneously [92]. Non structural proteins (NS) are involved in RNA binding activities enabling viral replication, and some newly identified proteins have various functions such as induction of host cell death and modulation of host response during infection [93–98]. M2, NA, and HA are envelope associated proteins located in the lipid bilayer obtained from the host cell. M2 is a transmembrane proton-selective ion channel regulating internal pH in intracellular compartments containing endocytosed virions [99]. NA and HA are membrane anchored proteins extending out of the viral membrane and are involved in the initial contact and release from the host cell. The structure of the influenza virion is illustrated in Fig.4A.

The influenza virus binds epithelial host cells in the upper respiratory tract of humans. HA binds sialic acid (SA) residues on target cells leading to viral internalization by endocytosis. NA promotes access to the target cell by degrading mucus and optimizing HA-SA interactions by digesting decoy residues [101]. Acidification of the endosome lumen induces conformational changes in HA, leading to exposure of hydrophobic regions in HA that embed into the endosomal membrane [102]. The viral membrane fuses to the endosome and release internal genetic material that migrate to the nucleus for transcription and replication. Newly synthesized viral products associate and are transported to the cytoplasm where they are packed and enveloped in a cell membrane studded with HA,
Figure 4: Schematic illustration of the influenza A virion and infection cycle. A) The influenza virion consists of eight internal genes encapsulated by a lipid envelope embedded with HA, NA and M2 external proteins. B) The influenza infection cycle. The virus binds receptors on the surface of host cells and is endocytosed. The virus then merges with the endosomal membrane and internal components are released, transcribed, replicated, and translated. Finally, new viruses assemble at the plasma membrane and are released from the host cell. Adapted from Shi et al. [100]

NA, M1, and M2. New virus particles are formed and finally released by NA cleavage of SA residues at the site of the escaping viral particle. Newly formed viral particles can then spread and infect new cells (Fig.4B).

Hemagglutinin

HA and NA are highly abundant on the influenza virus surface, where HA constitutes approximately 80% of the surface proteins and the remaining 20% is mostly NA [103,104]. HA is the initial mediator for viral binding and entry to host cells, and a main target for neutralizing antibodies. Antibody responses against NA have also been shown to restrict viral invasion, either by neutralization or by limiting release of newly synthesized virus [105]. Both HA and NA are thus under strong selective pressure exercised by the neutralizing antibodies of the immune system. Point mutations, or altered glycosylation patterns in immunogenic regions of HA mediate escape from the host immune system.

HA forms a trimeric spike in the viral membrane. Each monomer consists of a membrane
bound elongated stem and a globular head (Fig. 5). HA is synthesized and inserted into the ER as a glycopolypeptide precursor, HA0, in host cells during an influenza virus infection. HA0 polypeptides associate and form trimers before transportation to the plasma membrane through the Golgi apparatus. During transportation, HA0 undergoes extensive post-translational modifications before reaching the plasma membrane as mature HA of approximately 60kDa (unglycosylated) [106]. HA0 can be expressed, but infectivity depends upon proteolytic activation by cleavage of HA0 into two smaller fragments, HA1 and HA2, by host cell proteases. The fragments are held together by disulfide bridges. Acidification in late endosomes induces conformational changes in HA that results in exposure of the hydrophobic N-terminal of HA2, that embed into the endosomal membrane. Acetylation and oligomerization of HA are post-translational modifications that mediate and enhance viral formation and virulence. Fatty acetylation of HA is a prerequisite for proper viral budding and particle formation [107], and glycosylation is required for proper folding and transport of HA molecules [108]. Furthermore, glycosylation patterns impact the fitness and adaptation of the influenza virus. Due to the error prone replication of the viral genome, removal and addition of glycosylation sites happen frequently. However, most of the mutagenic changes appear in the head region, whereas the stem region is more conserved and contribute to the stability of the HA molecule [109]. As a method of viral adaptation, glycosylation sites can hide and impair antibody binding sites and neutralizing epitopes. Loss of oligosaccharides in proximity to the HA cleavage site can change accessibility of host proteases enhancing virulence in vivo [110]. Strain specific glycosylation patterns in HA have also been shown to activate innate receptors on human DCs in vitro [111].

Pandemic Influenza

As opposed to seasonal influenza that cause epidemics, certain influenza strains can be highly pathogenic and cause pandemic outbreaks. Outbreaks of an influenza pandemic can be devastating and the four pandemics in the last century claimed more than 50 million lives [80]. Pandemic influenza virus originates when influenza viruses of antigenic novelty enter the human population. Humans are then serologically naïve towards the new virus and have limited adaptive memory to defend against infections. Such novel influenza strains can arise from human adaptation of viral strains from avian or other animal sources.

HA binding to SA receptors is a main determinant for human adaptation of influenza viruses. The SA preference of influenza virions regulates infection, replication and shed-
Figure 5: Structure of 1918 H1 HA. A) HA associates into a homotrimer with a mushroom like shape. B) Structure of the HA monomer with indicated sites of receptor binding and cleavage between HA1 (orange and yellow) and HA2 (red). Made with PyMol Version 1.8, PDB 1RUZ [112]

ding, and thus transmission. Seasonal influenza virus preferentially binds saccharides terminating in SA α2,6-linked to galactose, while avian influenza strains have a binding preference for α2,3-linked SA. Epithelial cells in the upper respiratory tract in humans express α2,6-linked SA where seasonal influenza viruses are adapted to replicate and infect [113]. Epithelial cells of duck intestine are abundant in α2,3-linked SA, and avian influenza is adapted to infect cells with this phenotype [101,114]. The emergence of new influenza strains arises when viral strains adapt to infect α2,6-linked SA and pigs likely serve as a "mixing vessel" where genetic reassortment and viral adaptation are mediated. This theory was proposed in 1985 based on the understanding that influenza A strains do not easily transmit between humans and birds, but that the zoonotic barrier to pigs is rather low for both species [115]. Pigs display both α2,6- and α2,3-linked SA receptors in the upper respiratory tract, thus being susceptible to infections from both avian and human origin and serving as a molecular milieu for viral replication and antigenic reassortment [116]. New viruses with pandemic potential can thus arise from zoonotic infections where viruses from animal sources infect species that are known to be able to infect humans [117]. Pigs thus play a central role in pandemic emergence due to their
permissive infectivity to influenza strains of both avian and human origin (Fig. 6) [118].

Figure 6: Pig as mixing vessel for influenza A viruses. Aquatic birds are the natural reservoir for influenza A viruses. Domestic fowl can transmit virus from a wild life reservoir to pigs that facilitate adaptation and possible reassortment leading to human infectivity. Solid lines represent frequent cases of infection pathways and dotted lines represent possible but infrequent pathways. Adapted from Ma et al. [118].

Highly Pathogenic Avian Influenza

HPAIV strains H5 and H7, cause severe and often fatal disease in humans. Patients infected with HPAIV display high viral titers in lungs with development of pneumonia and multi-organ failures [119]. High replication efficiency and broad tissue tropism leading to systemic replication, are among the pathogenic determinants of HPAIV. Although few human to human transmissions have been reported and HPAIV generally exhibit poor infectivity and transmissibility in humans, it is clear that these viruses constitute a pandemic threat. WHO reported a mortality rate of 50% and 40% for influenza H5N1 and H7N9 through laboratory confirmed cases [120,121]. The majority of these cases were infected at live bird markets, vendors and breeding farms.

HPAIV have limited ability to infect and spread among humans, creating a zoonotic barrier for avian influenza viruses. H7 is unusual in its zoonotic potential as it has
been shown to be able to bind both α2,6- and α2,3-linked SA receptors on host cells and replicate in human epithelial airway cells [122–124]. Laboratory studies of H7 virus isolates from human infections show that the virus had accumulated few mutations indicating that H7 is approaching a breach in the zoonotic barrier [125]. Although the preference for α2,3-linked SA receptors limit human transmission, it may contribute to severe disease when virus does reach the human lower respiratory tract.

HPAIV differ from all other strains by a multibasic cleavage site (MBCS) in HA [126]. The acquisition of a MBCS in a low pathogenic strain does not transform the virus to a highly pathogenic strain, but the MBCS is a determinant of high pathogenicity [127]. Seasonal influenza virus is restricted to replicate in pulmonary tissue because the monobasic cleavage site in HA is only recognized by proteases in pulmonary tissue. In contrast, HA with a MBCS can be cleaved by the ubiquitously expressed proprotein convertase furin allowing the influenza virus to replicate systemically. Poultry infected with HPAIV often succumb due to damage to the central nervous system and virus have been detected systemically in fatal human cases [128,129].

Correlates of protection against influenza

Annual outbreaks of influenza with attack rates of 10-15% indicate that humans on average are infected every 10-20 years. These infections induce innate and adaptive immune responses and create immunological memory. Strain specific antibodies directed against the surface proteins HA and NA correlate with protection against influenza virus infection [130]. While anti-NA antibodies can limit shedding of virus, HA is the main target for protective antibodies that can inhibit viral entry and infectivity, and thus neutralize the virus [131]. Antibodies against HA can persist in serum for decades, and long lived plasma cells and memory B cells can remain the entire lifetime of an individual [42]. However, HA is an extremely polymorphic protein, and can vary up to 60% in amino acid sequence between different influenza A virus isolates [132]. Pressure from host immunity in combination with the lack of proof-reading during replication, drives selection of functional point mutations that mediates escape from host immunity. Antibodies are typically sub-type specific, with limited protection against other sub-types [133]. Furthermore, the immune system will preferentially utilize immunological memory based on previous infections, leading to generation of antibodies that cross-react against viruses of older infections, at the expense of development of specificities for determinants unique to new viruses [134,135]. This phenomenon is termed "original antigenic sin", and can hamper development of protective immunity, particularly at an older age.
Neutralizing antibodies against HA typically recognize sites adjacent to the receptor binding site. Antigenicity studies have pointed out five immunodominant antigenic regions in the globular head of H1 and H3 HA subtypes that are close to the receptor binding site [136,137]. These sites are hypervariable regions prone to antigenic drift. Point mutations in this region lead to escape variants and can have positive or negative effects on the receptor binding site affinity and specificity [138,139]. The receptor binding site in HA is a highly conserved shallow pocket at the distal end of the protein (Fig. 5), and is masked by highly immunogenic loops and glycosylation patterns [140]. This leads to a limited humoral response directed towards the receptor binding site. However, neutralizing antibodies binding the receptor binding pocket by insertion of a CDR loop contacting the conserved residues have more recently been identified [141–144]. Such antibodies are not strain specific and can cross react with multiple HAs and neutralize strains from multiple subtypes of influenza A virus.

In contrast to the highly variable head region of HA, the HA stem is highly conserved. It is also physically masked and glycosylated, effectively shielding it from the host humoral immunity. During an influenza infection, stem directed antibodies are formed, but the titers are low. These antibodies have been shown to recognize HA from various strains of influenza and can thus have a broadly neutralizing capacity [145]. Stem directed antibodies might not be neutralizing by directly blocking binding to host cells, but can inhibit structural rearrangements necessary for viral entry, such as membrane fusion after endocytosis [146,147]. Anti-stalk antibodies have also been shown to interact with Fcγ receptors, mediating antibody dependent cell cytotoxicity (ADCC) of infected cells [148]. Antibodies that recognize conserved regions of HA, such as the stem or receptor binding site, are of great interest in the development of universal influenza vaccines. Globular headless HA mini-stem, or other synthetic HA stem fragment immunogens that induce stem directed immune responses, can confer protective efficacy in mice and larger animals [149–151].

The main antibody isotypes raised during an influenza infection are IgM, IgA, and IgG. IgM antibodies are produced at the initiation of the immune response during primary infection, and provide initial protection via complement activation [152, 153]. Mucosal IgA can be produced and transported via trans-epithelial transmission to the site of infection, and neutralize virus locally [154]. Mucosal tissue represents the point of entry for influenza viruses. In these areas IgM and IgA antibodies are most prominent after infection, although IgG can also transude into the respiratory tract [155]. Serum IgG is a well known correlate of protection against influenza virus and can confer long term protection [42]. IgG levels rise after primary infection and peak around week 6, while IgM and IgA levels decrease after around two weeks post infection. Furthermore, IgA
responses are less frequent and of lower titers than IgG and IgM in most individuals [154]. During secondary infections, IgA and IgG are rapidly formed in serum and IgA in the mucosa [156].

**Vaccination against influenza**

Prophylactic vaccination has been the most effective way to diminish influenza related mortality and morbidity, and is one of the most cost-effective and life-saving inventions in history. Many vaccines have been developed empirically and the most successful influenza vaccines used today are attenuated or inactivated pathogens mimicking a natural infection [157]. Inactivated influenza virus vaccines are either whole virus vaccines or “split” vaccines with sub-virion particles prepared by chemical disruption of the viral membrane in order to reduce reactogenicity [158]. Effectiveness of seasonal influenza vaccines varies with the strain from 30-70% and a single vaccination is sufficient to establish life long immunity [159]. Importantly, studies of influenza vaccination in the U.S. for the 2005/06-2013/14 seasons estimated that over 40 000 deaths have been averted due to vaccination [160].

Because of the strong antigenic drift in influenza viruses, new vaccines have to be prepared every season. Based on surveillance data, WHO recommends influenza strains for the annual update twice a year, once for the northern hemisphere and once for the southern hemisphere. The vaccine virus strain is then selected, inserted, and propagated in embryonated chicken eggs before inactivation and vaccine deployment [161]. This process can take up to 11 months with a best case scenario of 6-8 months for vaccine production. The selection of a vaccine strain is thus based on the surveillance data rather than identification of the current antigenic structure of the season. This strategy has proven mostly successful for seasonal epidemics, but recent pandemic and sporadic zoonotic outbreak of HPAIV has illustrated that traditional vaccine manufacturing is insufficient in the face of a pandemic threat [162,163].

For prophylaxis in the face of a pandemic outbreak, vaccination is one of the most effective measures [164]. Stockpiling of pandemic vaccines could potentially offer protection, but requires that there are antigenic similarities between the vaccine strain and pandemic strain. Given the variability of influenza viruses, this is not likely to be the case. Additionally, the vaccination time line, i.e. start of the campaign and vaccine availability, in the event of a pandemic outbreak is crucial and important for global relief [165].

Novel vaccine technologies are necessary to limit the threat from highly pathogenic influenza strains with a pandemic potential. There are two main strategies to make such
pandemic vaccines. Firstly, a universal influenza vaccine that confers full or partial protection against virtually all relevant influenza strains by targeting conserved epitopes in the antigenic structure of the virion can be developed. Experiments have demonstrated that antibodies targeting the conserved stem region or the receptor binding site, can offer neutralization across strains and are referred to as broadly neutralizing [166, 167]. Further, vaccines can target conserved epitopes in NA or M2, or induce cellular immunity against conserved epitopes [168, 169]. Secondly, one could develop a vaccine format that rapidly could be produced to counter an emerging pandemic. Such a strategy would allow induction of neutralizing antibodies against the pandemic strain, potentially conferring sterilizing immunity.

DNA vaccines

DNA vaccines are a promising strategy for creating vaccines that need to be rapidly produced in a way that allows simple antigenic adaptation. The mutability of the influenza virus creates a continuous immunological game of cat and mouse. In order to counter the plasticity of the influenza virus, we need a matching plasticity of the vaccine platform. With recombinant DNA technology, new DNA vaccines encoding a new antigenic variant or an antigen from a new influenza strain can easily be produced.

The first paper to demonstrate that an immune response could be elicited against an antigen encoded by directly injected plasmid DNA was published in 1992 [170]. The next year, Ulmer et al. published a paper where mice were protected in a heterologous influenza A challenge after intramuscular injection of plasmid DNA encoding influenza A NP [171]. DNA vaccines induce both humoral and cellular immune responses, but are particularly effective in inducing CTL, likely because of the intracellular introduction of antigens translated from DNA.

DNA vaccines can be rapidly produced and deployed, potentially within weeks of a pandemic outbreak [172]. Due to the ease of chemical DNA synthesis, antigenic variants or perfect structural matches to wild type antigen can be used. In traditional egg based vaccine manufacturing, certain modifications have to be made to allow work with highly pathogenic strains of influenza. HPAIV usually kill embryonated chicken eggs so the MBCS has to be removed, and internal genes substituted to render the vaccine strains safer and optimize production [161, 173]. DNA vaccines thus offer a vaccine platform in which beneficial variants or exact matches can be produced with relative ease.

No live pathogens are involved in the manufacturing process of DNA vaccines, therefore,
such vaccines pose a very safe alternative for prophylactic vaccination. Additionally, DNA vaccines can be delivered without adjuvant to the dermis by non-invasive needle-free jet delivery systems [174,175]. Studies have indicated that the risk of incorporating foreign DNA in to the host genome is very low [174].

DNA vaccines elicit potent immune responses in mice, but are less efficient in larger animals where a prime-boost vaccination schedule with high doses is often necessary to elicit a response [176]. Novel DNA vaccine formulations can increase immunogenicity of DNA vaccines, which is particularly relevant in larger animals and humans [172,175,177,178].

APC-targeted vaccines

The immunogenicity of subunit vaccines can be increased by targeting of antigen to surface receptors on APCs. The goal is to be able to use synthetic peptides or recombinant proteins without the use of adjuvants, which are typically required to induce an effective immune response [179]. Kawamura and Berzofsky demonstrated increased immunogenicity after vaccination with antigen coupled to anti-IgG or anti-IgM in 1986 [180]. Others have later demonstrated increased immunogenicity following vaccination with antigen targeted to various receptors on APCs. Targeting moieties used to target antigen can be chemokine ligands for receptors on APCs [178,181–186], antibodies, Fab fragments, or a single chain variable fragment (scFv) specific for a receptor on APCs [77,177,187–198], moieties that bind natural triggers of innate immunity such as TLRs or macrophage scavenger receptors [199–206], or immunogens mimicking immune complexes targeted to complement or Fc receptors [207,208].

The type of surface molecule that is targeted on APCs influence the type of immune response that is elicited [184,185,197,198,209–211]. For example, targeting Xcr1, a receptor expressed exclusively on cross presenting DCs [212], have been shown to skew towards a Th1/IgG2a response and increase cellular immunity [184]. In contrast, targeting MHCII molecules, which is expressed on a wide range of APCs, have been shown to skew towards a Th2/IgG1 response. Further, the interaction between the receptor on the APC and the targeting moiety of the immunogen affects the skewing of the immune response. This was demonstrated with hemagglutinin targeted to Xcr1 on cross presenting DCs in mice with either a human or murine version of the Xcl1 chemokine. The murine Xcl1 induce receptor mediated endocytosis of the immunogen and resulted in a strong cellular response, while the human Xcl1 did not induce endocytosis, and increased the antibody response against...
hemagglutinin in mice [185]. These observations open possibilities for the generation of a tailored vaccine that can polarize the immune response in favor of protection against the natural pathogen, and abolish the need for co-delivery with adjuvant [192].

**APC-targeted DNA vaccines against pandemic influenza**

Targeting of HA to APCs in mice has been shown to confer full protection against a lethal challenge with influenza virus [172, 209]. Targeting MHCII molecules have been shown to significantly increase antibody responses after vaccination and confers sterilizing immunity against H1 influenza in mice [172], while targeting APCs with the chemokine Mip1α have been shown to induce a more mixed response of antibodies and cellular immunity [209]. The increased antibody responses against HA have also been observed in larger animals with MHCII targeted DNA vaccination [175]. Induction of a strain specific neutralizing antibody response against influenza might be favorable for a pandemic preparedness vaccine, however cross priming of CTL can also be important in fighting viral infections. Importantly, the APC targeted DNA vaccine could be produced, and mice could be vaccinated in only 3 weeks after the HA sequence was available online in response to the outbreak of the 2009 H1N1 pandemic [172].

In these studies, plasmid DNA vaccines were delivered in dermis or muscle followed by electroporation of the injection site to increase protein expression (Fig. 7A). The DNA vaccines encoded a homodimeric vaccine protein that consisted of an antigenic unit with globular HA from PR8 and a targeting unit that consisted of the chemokine Mip1α [209], or a scFv specific for MHCII (I-E<sup>d</sup>) [172,175] linked together with a dimerization domain consisting of a human CH3 domain from IgG3 (Fig. 7B).

The immune potentiating effect observed when targeting receptors on APCs have been proposed to be a function of increased uptake, processing and presentation of antigen by APCs [213]. Depending on presentation pathway, which is affected by the targeting unit, this can induce augmented T helper cell levels, or induce presentation on MHCI resulting in cross priming of cytotoxic T cells (Fig. 7C).

The work presented here builds on these observations and presents an APC targeted DNA vaccine candidate against pandemic influenza. Targeting antigen to APCs is studied in detail in order to increase knowledge necessary to create novel vaccines as preparedness for a potential outbreak of pandemic influenza.
Aims of the Study

HPAIV constitute a pandemic threat. Traditional vaccine manufacturing can not properly meet this threat, and new and more efficient vaccines are necessary. Earlier studies using the APC-targeted DNA vaccine format against H1N1 influenza viruses indicated that mice [172] and larger animals [175] could be protected against influenza challenge after vaccination. Importantly, the vaccines could be rapidly produced, and induced an accelerated immune response as compared to non-targeted controls. Furthermore, targeting to APCs significantly increased immunogenicity and targeting towards MHCII molecules could favorably skew the vaccine induced immune response towards antibody mediated protection [209]. This is important as antibodies represent an important correlate of protection against influenza.

A first aim for this study has been to develop an APC targeted DNA vaccine against HPAIV H7. While this approach has proven successful for H1N1 influenza viruses [172, 175], the underlying mechanisms for protection have not been fully elucidated. A second aim for this study has thus been to examine the immunological mechanisms associated with MHCII-targeting of antigen. Finally, since targeting of antigen to different receptors...
can differently polarize the immune response [184,185,197,198,209–211], it was of interest to see how a targeting unit could be modified to induce different types of immunity.

The aims of this study have been:

1. Construct and characterize a DNA vaccine against HPAIV (H7N1) *in vitro* as a candidate for vaccination against an emerging pandemic situation.

2. Investigate antibody and T cell responses in mice after vaccination with the vaccine against avian influenza, and see if the vaccine can protect against a lethal influenza challenge with H7N1 influenza.

3. Quantitatively examine B cell responses after MHCII-targeting of antigen in order to investigate the mechanisms behind protection.

4. Examine antigen specific T- and B cell collaboration in the presence of MHCII-targeted antigen in order to further understand how the immune responses were formed.

5. Make an HA probe able to detect vaccine induced GC B cells, and investigate the development and affinity maturation of antigen specific B cells from a naïve repertoire.

6. Investigate how a chemokine targeting unit can be modified to induce antibody mediated protection against influenza.
Summary of Individual Papers

Paper I
A DNA vaccine that targets hemagglutinin to antigen presenting cells protects mice against H7 influenza

Tor Kristian Andersen, Fan Zhou, Rebecca J. Cox, Bjarne Bogen, and Gunnveig Grødeland.

In press, Journal of Virology

Due to a prolonged production time, conventional vaccine manufacturing is unlikely to counter a pandemic outbreak. Both the 2009 H1N1 pandemic and the seasonal H7N9 epidemics in China demonstrate that a novel pandemic vaccine candidate is urgently needed. This paper presents a DNA vaccine that targets HA from HPAIV H7N1 to either MHCII or the chemokine receptors 1, 3, and 5 expressed on APCs in mice. The paper focuses on early responses after immunization since the vaccine aims to serve as an immediate barrier to a potential pandemic, and results showed that APC-targeting of antigen significantly increased antibody titers at week 5 post vaccination. The antibodies were confirmed neutralizing in an H7 pseudotype neutralization assay. Further, a cytotoxicity assay showed that CTL were induced, and that these contributed significantly to reduced morbidity. However, T cell depleted mice did not have a significantly increased mortality as compared to sham depleted mice, indicating that antibodies alone could confer protection. Importantly, mice immunized with APC-targeted vaccines were fully protected against a lethal challenge with H7N1 influenza virus, while non-targeted controls demonstrated significantly higher morbidity and mortality.

Paper II
Targeting of antigen to major histocompatibility complex II accelerates the germinal center reaction

Tor Kristian Andersen, Peter Huszthy, Ramakrishna P. Gopalakrishnan, Johanne Jacobsen, Marte Fauskanger, Anders Aune Tveita, Gunnveig Grødeland, and Bjarne Bogen.

Submitted manuscript

Targeting of antigen to MHCII increases immunogenicity after a single vaccination. Previous data have demonstrated an increase for different antigens and in different species of animals, and also demonstrated that MHCII-targeting is associated with induction of a Th2 type of immunity. This paper is a mechanistic study of the responses induced after immunization with antigen targeted to MHCII.

Experiments were performed with a scFv containing the λ2315 idiotypic antigen and id-
Iotope specific T and B cells in combination with congenically marked BALB/c mice. *In vitro* experiments demonstrated that targeting of antigen to MHCII lead to an increased presentation of idiotypic peptide-MHCII complexes on professional APCs. Furthermore, MHCII-targeting of antigen increased the efficacy of T-B cell collaboration, and demonstrated equal proliferation to a non-targeted control vaccine at a 10-100-fold reduced dose. *In vivo* transfers of Id specific T and B cells in combination with protein immunization demonstrated similar trends, and MHCII-targeted antigen was found to significantly increase T and B cell proliferation. In addition, MHCII-targeting significantly accelerated development of GC B cells and follicular T helper cells. Further, an experiment with T and B cell transfer in immunodeficient NOD scid gamma mice demonstrated that the increased antibody response could be mediated by antigen specific T and B cells alone, without additional APCs.

To generalize these observations with a wild type system, we demonstrated that DNA vaccines targeting HA (PR8) to MHCII could induce more antigen reactive GC B cells with a higher avidity than non-targeted control vaccines. In support of this finding, higher numbers of IL-4 secreting cells were found in the LNs, and ultimately a higher number of anti-HA secreting plasma cells in the bone marrow, after immunization with MHCII targeted HA as compared to the non-targeted control vaccine.

**Paper III**

*An endocytosis deficient murine Xcl1-fusion vaccine enhances protective antibody responses in mice*

Arnar Gudjonsson, Tor Kristian Andersen, Vibeke Sundvold-Gjerstad, Bjarne Bogen, and Even Fossum.

*Manuscript*

Targeting antigen to surface receptors on DCs can increase immune responses against subunit vaccines. Furthermore, the type of target receptor can influence the polarization of the immune response. Previous data, comparing a human and murine Xcl1-fusion vaccine that target murine Xcr1+ DC with the Xcl1 chemokine, demonstrated that the interaction between the receptor and vaccine protein affected the immune response. The human Xcl1-fusion vaccine induced significantly stronger antibody responses and it was found to be related to the lack of receptor mediated endocytosis of the human chemokine.

The use of foreign Xcl1-fusion vaccines is undesirable when translating this observations to human or veterinary medicine due to potential cross-reactive responses against the chemokine. We have thus identified a mutant version of murine Xcl1, labeled Xcl1(2-93)
due to removal of a conserved valine in position 1 of the mature chemokine, that retains specific binding to Xcr1+ DCs without inducing endocytosis of the receptor.

DNA immunization with Xcl1(2-93) conjugated to influenza hemagglutinin (HA) induced stronger antibody responses, with higher end point titers of IgG compared to WT Xcl1-HA. Xcl1(2-93) also resulted in increased numbers of HA reactive germinal center B cells with higher antigen avidity. The WT Xcl1-HA vaccine induced stronger T cell responses. Serum transfer experiments indicated that Xcl1(2-93)-HA induced antibody responses that provided better protection against influenza infection compared to WT Xcl1-HA. In sum, the observations indicate that a mutant Xcl1 can easily be translated to a human or veterinary vaccine setting. Finally, targeting antigen to Xcr1+ DCs in the absence of endocytosis enhances antibody responses.
Methodological considerations

DNA and protein vaccines

All vaccine constructs were cloned into the pLNOH2 plasmid under an hCMV promoter. The pLNOH2 plasmid was originally constructed to express Iggs [214], and as the originally developed APC-targeted DNA vaccines consisted solely of Ig derived sequences, this represented an appropriate expression vector [177]. Restriction cassettes have been incorporated into the pLNOH2 to allow for easy substitution of antigenic or targeting units. Thus, new antigens can simply be amplified, flanked with the appropriate restriction sites, and inserted into the vaccine format. Even though pLNOH2 was optimized for expression of Ig sequences, insertion of scFv or chemokine sequences into the V region and antigenic units (HA or scFv sequences) into the C region resulted in proper secretion. Interestingly, the plasmid has later been proven to have a similar efficacy as other plasmids developed specifically for DNA vaccines (Grødeland et al, unpublished).

Insertion of HA from influenza H7N1 [A/turkey/Italy/3889/1999 (H7N1)] was based on previous work with H1 influenza viruses (PR8 and Cal07), where aa 18-541 were inserted [172, 215]. Thus, the globular head region of HA was included, as well as a part of the membrane embedded stem region. Here, we inserted the corresponding H7 sequence (aa 19-536) into the vaccine plasmid. However, the potential for T cells to cross react between H7 and seasonal influenza strains in humans is poor, and T cell epitopes cannot be directly compared between the strains [216].

For several key experiments in the mechanistic study of MHCII-targeting of antigen (paper II), the vaccines were delivered as proteins since it was essential that equimolar doses of MHCII-targeted and non-targeted control vaccines were used. Protein vaccines will allow for a direct assessment of antigenic contents, as opposed to DNA vaccines, and the only difference between MHCII-targeted vaccines and non-targeted controls would be the specificity of the targeting moiety. For a DNA vaccine, small differences in secretion efficacy after vaccination could have influenced the observed differences between targeted and non-targeted vaccines.

For validation of vaccine protein integrity, the purified proteins were examined by polyacrylamide gel electrophoresis and Coomassie staining. Furthermore, standardized enzyme-linked immunosorbent assays (ELISAs) were run to characterize purity and, most importantly, make sure that the molarities of the batches were identical. In summary, we
ensured that equal amounts of antigen were used in key assays for comparisons between MHCII-targeted and non-targeted vaccine proteins.

**Antibody responses in ELISA**

Antigen-specific antibodies induced by immunization were detected in ELISA against recombinant HA from influenza H7N1 [A/Shanghai/1/2013 (H7N9)], H1N1 [A/Puerto Rico/8/1934 (H1N1)], or M315 monoclonal antibody containing the λ2^{315} idiotypic antigen. ELISA was also used to detect antigen-specific IgM production in *in vitro* T-B collaboration assays. ELISA is a rapid and sensitive way to quantify the presence of a specific protein, such as antibodies in serum. The ELISAs were performed in a capture assay sandwich in which the protein of interest can be sandwiched between a capture antigen and detection antibody. Thus, two characteristics can be investigated in a single assay, such as antibody specificity and isotype. Idiotope-specific antibodies of IgM and IgG isotypes were detected in ELISAs with standardized samples and a sensitivity of 10ng/ml was determined based on the standard curves. The sensitivity limit was defined as the last serum dilution with a value higher than the mean of a similar dilution from NaCl or PBS vaccinated mice, added 5 times the standard error of the mean. Endpoint titers were calculated by the same method in non-standardized ELISAs with recombinant HAs as capture antigens. ELISAs will provide quantitative knowledge about the levels of antibodies induced after vaccination, but will not give qualitative information about the induced antibodies.

**Measurement of neutralizing antibodies**

Conventional serological analysis such as hemagglutinin inhibition (HI) tests are relatively insensitive to the detection of human antibody responses against avian HAs [217]. Due to mixtures of α2-6 and 2-3 linked SA receptors on the cell surfaces of different species, HA from avian influenza does not effectively agglutinate erythrocytes from human, chicken and turkey, but a modified HI assay can be used with horse erythrocytes rich in α2-3 linked SA [218]. Furthermore, work with avian influenza viruses will require BSL-3 or higher. For this reason, we have not examined sera after vaccination in an HI assay.

To allow a safer assessment of neutralizing antibodies after vaccination with vaccines against avian influenza, lentiviral pseudotypes expressing heterologous glycoproteins have been developed [219]. Lentiviral pseudotypes undergo a single infectious cycle and do
not spawn new viruses, and assays including these viruses do not require BSL-3 facilities. Moreover, the pseudotype virus has been generated with a luciferase reporter gene, and infectivity can readily be detected as an increase in luciferase levels. Here, we have thus used a pseudotype neutralization assay for a qualitative assessment of the vaccine induced antibody responses (paper I). The pseudotype neutralization assay, in combination with examinations of lung pathology after viral infection, will provide a good indication of the neutralizing properties induced by vaccination.

**ELISPOT assay**

Enzyme linked immunosorbent spot (ELISPOT) assays were used to quantify cells secreting either cytokines or antibodies [220]. The assay allows the identification of cytokine or antibody secreting cells at the single cell level. However, in contrast to an ELISA, ELISPOT does not give any information about the amounts of cytokine or antibody produced.

For an assessment of plasma cells secreting antigen specific antibodies, bone marrow suspensions were seeded on membranes coated with recombinant HA from influenza A/Puerto Rico/8/1934 (H1N1). This method allows quantification of long lived antigen specific plasma cells in the bone marrow after vaccination.

In order to quantify T cells secreting either interferon gamma (IFN-γ) or interleukin 4 (IL-4) ELISPOTs were set up with splenocytes or lymphocytes added on top of membranes coated with an antibody against the particular cytokine. The lack of characterized BALB/c MHC class I and II restricted peptides in HA from H7N1 influenza made it necessary to stimulate the splenocytes with full length HA in the IFN-γ ELISPOT (paper I). In contrast, for measurements of IL-4 secretion after vaccination with HA from H1N1 influenza (paper II), known BALB/c MHC class I and II restricted peptides could be used [215, 221, 222]. The advantage of using individual peptide stimulation is that the binding preference for MHC class I and II is known. This allows CD4⁺ and CD8⁺ T cell responses to be specifically addressed, typically associated with helper and cytotoxic effector functions, respectively. Stimulation with full length HA will represent all epitopes and measure both CD4⁺ and CD8⁺ T cell responses. Still, because stimulation with a selected peptide might exclude some activating epitopes, full length protein was always included as control.
Measuring cytotoxic T-lymphocytes

We wished to associate the IFN-γ secreting cells with CTL responses. Because the IFN-γ ELISPOT was performed with full length H7 protein, CD4+ T cell responses were also detected and an increased CD8+ CTL response could not be directly concluded from those results. To specifically address CTL responses, a mouse lymphoma A20 B cell line expressing cytosolic H7 [A/turkey/Italy/3889/1999 (H7N1)] with a GFP reporter and an A20 control expressing mCherry was generated. The cell lines were made by retroviral transduction, and high expressing cells were selected by FACS. The cell lines were then characterized by flow cytometry for proper expression of reporter proteins after expansion, and H7 was detected in cell lysates by Western blotting. By injecting equal amounts of these cell lines intravenously in immunized mice and calculating the ratio between the two in the spleen 16h later, the H7 specific CTL response could be evaluated. The A20 cell lines with fluorescent reporter genes will present foreign peptides derived from the fluorescent proteins, but a primary cytotoxic response against these epitopes is unlikely during the 16h experimental window. Further, by calculating the ratios between the H7 and control cell lines, and assuming similar unspecific killing or death for the two cell lines used, the killing ratios are likely mediated by antigen specific CTL responses. For globular antigens with unknown MHCI-restricted peptides in the particular species of research animal, this method offers a quantitative and functional measurement of CTL responses.

Influenza challenge in mice

Mice are not a natural host for influenza viruses, but most strains can be adapted to be virulent in mice by multiple lung to lung passages [223–225]. The A/turkey/Italy/3889/1999 (H7N1) low pathogenic avian influenza strain was kindly provided by the National Institute of Biological Sciences (NIBSC). The strain was adapted to mice for use in challenge experiments, and the virus successfully developed into a virulent phenotype after approximately 10 passages in mice. Weight loss was used as a clinical readout for disease in these experiments, and 20% weight loss was defined as the humane endpoint. Mice were inoculated with titrated doses of mouse adapted H7N1 virus in order to determine LD50 by using the Reed Muench method [226].

Mice show several signs of disease during an influenza infection, such as a hunched back, ruffled fur, reduced activity and hind limb paralysis. A clinical score sheet can be used to score morbidity observed in mice, but can be a fairly subjective approach. Measuring
weight loss is an efficient and objective method to evaluate morbidity, and was thus used in all challenge experiments. To secure animal welfare and minimize disease burden while still addressing morbidity in viral challenge experiments, mice were terminated at 20% weight loss. This is in accordance with guidelines of the Norwegian Animal Research Authorities.

**Idiotype-specific T and B cells**

T and B cells specific for the idiotypic (Id) antigen $\lambda^{\alpha315}$ from Id positive M315 were used to investigate antigen specific T-B collaboration. Naïve Id specific T- and B cells were enriched from spleens from TCR transgenic- [227] and anti-Id BCR double knock in mice [228]. Thus, naïve antigen specific T- and B cells could be studied in detail in multiple assays *in vitro* and *in vivo*. Antigen specific cell numbers could be controlled, and proliferation assays and detailed tracking of cellular development could be performed in a way that would be difficult with wild type systems.

The affinity of the BCR of the anti-Id+ B cells is likely affecting the sensitivity of the system. The BCR has an intermediate affinity for the antigen ($[\text{Ka}] = 0.77 \times 10^7 \text{ M}^{-1}$ [228]), but is likely high compared to the affinity of practically any wild type B cell. B cells with increased antigen affinity are more easily activated and might be more potent in forming GCs. In a wild type system, many T and B cell clones will likely recognize the antigen and expand. As such, T and B cells from multiple lineages can together establish an immune response and offer different ways of recognizing the antigen and form a broader memory repertoire. In the antigen-specific system used here, it is appropriate to interpret the observed responses as monoclonal T and B cell responses to the antigen.

NSG mice are severely immunocompromised and may be readily engrafted with cells from other mice mouse strains or humans. By transferring antigen specific T and B cells to NSG mice as opposed to BALB/c, the contribution from other APCs could be addressed. NSG mice lack relevant host APCs that can stimulate the transferred T and B cells. The response in NSG mice must be assumed to result solely from T-B collaboration of transferred cells.

Direct control of the numbers of antigen specific T- and B cells are a great benefit in these assays. Some of the events we aimed to investigate were rare, such as the presence of early plasma cells in the spleen after immunization. A relatively large number of antigen specific cells had to be transferred to the host to detect appropriate numbers in flow cytometry,
which could not have been performed with similar accuracy of antigen specificity in a wild
type system.

To generalize the observations made with the Id-specific system, we performed similar
experiments with HA antigen and wild type mice. This is important in order to establish
that the accelerated GC formation observed with the Id-specific system, was not simply
due to the presence of high numbers of antigen specific cells, but could be observed with a
viral antigen and wild type mice. The same results were obtained in the wild type system
enhanced the conclusions drawn from both methods.

Proliferation assays

*In vitro* proliferation assays were performed by measuring incorporation of radiolabelled
thymidine. In these proliferation assays, either the T or B cells were inactivated by
irradiation. The inactivated cell thus functioned as a non-proliferating feeder cell, while
proliferation of the other cell type could be measured. The inactivated cells were always
incubated alone in the presence of LPS or ConA as a control to ensure that they could not
proliferate. Incorporation of radiolabelled thymidine is a sensitive assay that can easily
be performed with a large number of samples and was thus used to characterize many of
the essential mechanisms in the T-B collaboration assay before continuing with *in vivo*
experiments. While thymidine incorporation gives a qualitative measure of proliferation,
irradiation of one of the cell types could diminish T and B cell proliferation. We therefore
employed celltracer assays that allow direct identification of cell division and activation
markers in flow cytometry. However, celltracer dyes are lost during cell division and thus
restricts the identification of dividing cells to 6 or 7 generations. Thymidine incorporation
assays in combination with flow cytometry measurements of celltracer dyes and activation
markers give both a qualitative and quantitative measure of proliferation and activation
of cells.

*In vivo* proliferation was measured by incorporation of BrdU. BrdU incorporated into
cellular DNA can then be detected by antibodies in flow cytometry. Mice were given
continuous BrdU so that proliferating cells would incorporate BrdU into newly synthesized
DNA during the entire experiment. This allows tracking for a longer period of time
because signal is always added to proliferating cells. Some toxicity has been noticed with
BrdU feeding over longer periods of time (>1 week), but BALB/c mice are less affected by
BrdU [229] and no adverse effects were observed in the BALB/c mice receiving continuous
BrdU for experiments lasting up to 2 weeks.
Identification of GC T and B cells

Recruitment and expansion of antigen reactive B cells in GCs is a prerequisite for the establishment of T cell dependent humoral immunity. The development of Id-specific T and B cells could be tracked in vivo after transfer to CD45.1 congenic BALB/c mice. Thus, transferred T and B cells could be detected based on CD45.2, B220, and CD4 surface markers and any modifications or down regulation of the T- or B-cell receptor would not affect the detection of the antigen specific cells. Thus, T cells that developed into helper cells that might down regulate the TCR and up-regulate CD40L could still be identified in the antigen specific system. B cells that developed into plasma cells or that had modified the BCR could also be identified and quantified.

It was important to generalize the development of GCs in the Id specific system to a wild type setting as the presence of high numbers of antigen specific T and B cells could affect the sensitivity of the system. Thus, DNA vaccination with APC-targeted HA antigen was performed in BALB/c mice. In order to identify GC B cells that were reactive to the immunogen, an HA probe was developed with HA from PR8. The probe had a six histidine tag at the carboxy terminal, and could be detected in flow with an anti 6x histidine monoclonal antibody. In order to reduce background binding, tyrosine at position 98 was exchanged for phenylalanine (Y98F) to abolish non-specific binding of HA to SA receptors [230]. It was important to characterize the binding of the probe to SA receptors, which was found negative, and to a wild type B cell repertoire. A wild type B cell repertoire will demonstrate antigen binding at high concentrations due to the nature of the germline encoded BCRs that may have weak affinity for virtually any antigen [231]. By characterizing the non-specific binding to B cells, the antigen specific GC B cells raised by immunization could be investigated. Additionally, with a defined background level, titrations with the probe could indicate the avidity of the GC B cells as high affinity B cells would be stained with lower amounts of antigen.

Finally, germinal center structures were investigated with immunofluorescent staining of cryopreserved spleen or LN sections. While flow cytometry gives quantifiable single cell data, it does not give information about the structure and physiology of the germinal centers in the organs. Immunohistochemistry of sections from spleen or LN can show the structural organization of the GC and help identify the absolute numbers of GCs and their size. Importantly, similar conclusions could be drawn from data collected with flow cytometry and immunohistochemistry, thus complementing the observations made with the two techniques.
Animal health and welfare

All mice were kept at the Department of Comparative Medicine at Oslo University Hospital. All experiments and breeding of mice, except work involving live virus, was performed at the minimal disease unit. Mice were regularly tested for pathogens according to the Federation of European Laboratory Animal Science Association (FELASA) guidelines for health monitoring of laboratory animals. All personnel involved with research animals had passed FELASA level C course for laboratory animal handling. Work involving live virus was performed at the infectious unit at the Department of Comparative Medicine under biosafety level 2+ conditions. Mice were immunized in the minimal disease unit and transferred to the infectious unit at the start of the challenge experiment.

General Discussion

Promising DNA vaccines targeting antigen to APCs in mice [172] and larger animals [175] demonstrate that there is a clear potential for such vaccines to serve as a first barrier against pandemic influenza. DNA vaccines can be rapidly modified and produced, and HA from an emerging pandemic strain can be rapidly inserted to counter a pandemic. At present, the world is facing threats from yearly epidemics of HPAIV H7N9 in China. Such epidemics started in 2013, but an all-time peak took place early in 2017, indicating that the virus is spreading [232, 233]. Although limited human to human transmissions have been observed, some human isolates have demonstrated binding to both avian and human SA receptors. Thus, there has already been a breach in the zoonotic barrier [101, 122, 123, 234, 235]. A vaccine candidate that can be rapidly produced, and that does not rely on egg based production, is urgently needed in preparation for an avian influenza H7 outbreak.

DNA vaccination

DNA vaccines can overcome many of the challenges facing the establishment of a vaccine platform for pandemic preparedness. The vaccines can be easily developed and modified, and can be produced using a generic production line. DNA is stable over a wide range of temperatures, making a dedicated cold chain redundant [236]. There is, however, still some safety concerns in the public related to the use of DNA vaccines. Introduction of foreign genetic material introduces an intuitive risk of undesired integration into the host.
genome. However, studies have indicated that this risk is very low [174], and clinical studies in humans have over the past decade demonstrated that DNA vaccines are safe [237–240]. That said, there is still no DNA vaccine licensed for use in humans. The reason is primarily that DNA vaccines are poorly immunogenic, and typically requires extended prime/boost schedules. Many methods for increasing the immunogenicity has been developed, such as optimization of transcriptional elements e.g. by the use of human CMV promoter [241], modifications of the Kozak consensus sequence [242], and codon optimization [243]. DNA delivery with electroporation or gene gun systems can also increase DNA uptake and protein expression in addition to induction of cytokine influx in the local environment effectively acting as an adjuvant [175, 244, 245]. Due to public concerns, prophylactic vaccination on a seasonal basis might be out of the scope for DNA vaccines, but DNA vaccines represent a realistic platform for global relief in the face of an emerging pandemic.

DNA vaccines mimic live pathogens or live attenuated vaccines in the sense that all these strategies lead to production of immunogens in the host. The consequence is that immunogens are produced in their native form, and with all post-translational modifications intact. Furthermore, protein production in the host cell leads to presentation of antigenic fragments on both MHCI and MHCII molecules. As such, it allows for broader recognition by the immune system and also potentially a broader immune response.

Delivery of DNA encoded immunogen results in a gradual increase in antigen produced by host cells and drained to LNs. Gradual increase of antigen delivered to LNs have been demonstrated to increase the GC reaction compared to one or multiple bolus injections of antigen [246]. During a natural infection, antigen that is drained to the LNs is likely to gradually increase during the first days of infection, and DNA vaccination might mimic this form of antigen delivery to the LNs. After an immune response has been established, antigen secreting cells will be removed by the antigen specific immune response, thus ensuring that immune priming will appropriately be terminated once immunological memory is established.

**APC-targeted DNA vaccines for pandemic preparedness against H7 influenza**

In conventional vaccine design, highly pathogenic viruses must be adapted for production in chicken eggs [161]. For HPAIV, these modifications involve removal of the MBCS and exchange of some internal genes. Removal of MBCS in HPAIV can compromise immuno-
genicity [126,247,248], and we correspondingly demonstrated that an H7 antigen with intact MBCS resulted in a faster antibody response as compared to an antigen where the MBCS had been removed (paper I). No significant difference in expression was observed between the two constructs. The ELISA pair antibodies used to detect secreted protein also indicated that they are properly folded. However, it is likely that the HA with an intact MBCS can adopt a structure more similar to native HA, potentially exposing immunodominant epitopes. Because HA with MBCS can be cleaved by ubiquitous intra- and extra-cellular proteases the vaccine proteins are likely produced in vivo with monomeric HA1 and HA2 bound to the targeting complex, while HA without the MBCS are expressed as a single HA0 polypeptide bound to the targeting complex.

In previous work with DNA vaccines targeting H1 antigen to APCs, the response was very low after vaccination with the non-targeted control in mice [172]. To our initial surprise, the H7 antigen was more immunogenic and could raise moderate antibody levels in ELISA after non-targeted DNA vaccination. The in vitro expression levels of the DNA vaccines were investigated in 293E cells, and demonstrated a somewhat higher expression of non-targeted control vaccines (Paper I). However, the variations were within tolerable levels expected from using DNA as vaccine format. Although we assume that expression levels are similar in vivo, this is likely affected by delivery method and tissue [249]. Increased immunogenicity of the avian HAs might originate from differences in glycosylation patterns and binding preference to SA receptors between human and avian influenza strains. These factors have been shown to induce production of pro-inflammatory cytokines in human DCs [111,250] and activation of innate-like γδ T cells [251]. Avian influenza antigens might therefore be more immunogenic in mice and the immune-potentiating effect observed when targeting antigen to APCs is less pronounced with avian HA antigens as compared to the H1 influenza antigens published previously [172,175,177]. Nevertheless, our experiments showed that only APC-targeted vaccines could confer full protection in BALB/c and CB6F1 mice after a single DNA vaccination followed by infection with a lethal dose of H7N1 virus (Paper I). In line with this, the pseudotype assay measuring the neutralizing potential in sera after vaccination demonstrated a clear difference between APC-targeted and non-targeted vaccines.

**Targeting of H7 to APCs**

Targeting of antigen to APCs is a strategy to raise immunogenicity, but can also favorably skew the immune response to a particular branch of immunity [184,185,197,198,209–211]. Previous data have demonstrated that targeting of antigen to MHCII is very efficient for
raising high titers of neutralizing antibodies, which is of particular interest for a pandemic influenza vaccine [172]. Targeting APCs with the chemokine Mip1α (Chemokine (C-C motif) ligand 3) that bind chemokine receptors 1, 3, and 5, has been shown to induce a dual activation of CD4+ and CD8+ T cell responses [178, 183, 209].

There are clear rationales for using both of these two targeting strategies to create a pandemic influenza vaccine. MHCII receptors expressed on professional APCs are the essential molecules for initiation of a humoral immune response. High titers of high affinity antibodies observed after targeting antigen to MHCII molecules (Paper II) is an essential goal of many influenza vaccines, where HA-specific neutralizing antibodies can mediate sterilizing immunity. Serum antibodies against HA is thus a well-established correlate of protection against influenza [252]. However, influenza viruses are highly variable, and because it is very difficult/impossible to predict exactly which influenza strain will pose the next pandemic threat, induction of a broader Th1/Th2 type of immune response might be preferable. Pre-existing CD8+ T cell immunity can enhance viral clearance and decrease morbidity in mice, even though it does not prevent infection [252, 253]. Targeting with the chemokine Mip1α raised a more mixed response and demonstrated lower titers of neutralizing antibodies (Paper I). However, targeting of H7 with Mip1α reduced morbidity, increased CTL, and showed a higher survival in T cell depleted mice as compared to mice receiving the MHCII-targeted vaccine.

Heterologous strains were used in the DNA vaccine and in the challenge virus (97% sequence homology) in most of the experiments. An additional experiment where the vaccine and challenge strain were homologous were performed to more directly address contribution from potentially neutralizing antibodies in vivo. While the MHCII targeted vaccine performed slightly better in the homologous challenge, the Mip1α targeted vaccine performed equally well in the two experiments. This indicates that non-neutralizing antibodies and CTL were essential in overall protection, especially in a situation where there is heterology between vaccination and challenge strain(Paper I). Non-neutralizing antibodies can induce Fc receptor mediated effector functions such as ADCC, which can be cross reactive [254] and may be of particular importance in a pandemic setting [255]. Additionally, non-neutralizing H7 specific antibodies have been demonstrated to be protective in mice [256,257]. The results with APC targeted H7 antigen thus points to Mip1α as a more appropriate and safer targeting unit for the development of pandemic vaccines.
Underlying mechanisms of APC targeting

Targeting of antigen to APCs can increase uptake and presentation on MHCI or MHCII molecules, inducing an enhanced immune response (Paper II). Utilization of the MHCI or MHCII pathway for inducing a Th1 or Th2 type immune response, respectively, is demonstrated with use of Xcl1 as targeting unit, selectively binding to Xcr1⁺ DCs that can cross present extracellular antigen to the MHCI presentation pathway in mice [258, 259]. Such targeting induces a strong Th1 type of response, due to rapid endocytosis of vaccine proteins ligated to Xcr1 and an ensuing presentation to MHCI [184, 185]. Interestingly, by exchanging the Xcl1 ligand with a mutant chemokine that does not induce receptor mediated endocytosis, the induced immune responses will shift towards humoral responses (Paper III). Targeting to Xcr1 on DCs thus offers a customizable platform where modifications of the ligand, such as receptor affinity and induction of downstream signaling and endocytic activation, can polarize the immune response. The very mechanisms behind induction and sorting of endocytosed chemokine receptors are not completely understood, but is dependent on effective phosphorylation affecting the downstream signaling and sorting pathway [260–263]. Although no direct association between internalization speed or receptor affinity and antibody responses has been discovered [264], ligating chemokine receptors with a mutated or xenogeneic chemokine that does not induce endocytosis is evidently efficient in raising antibody over CTL responses [185] (Paper III).

Xcr1 is exclusively expressed by cross presenting DCs [212]. By comparison, chemokine receptors 1, 3, and 5 and MHCII molecules are expressed on a wider range of immune cells. Mip1α is also efficient in chemotactic recruitment of leukocytes [178, 265] and can channel loading of internalized antigen to both MHCI and MHCII presentation pathways [266, 267]. CTL induced by cross priming require involvement of CD4⁺ helper T cells and, importantly, this helper cell population is only effective when both the helper T cell and CTL is primed by the same APC [268]. The dual presentation on MHCI and MHCII molecules by an APC can therefore induce an effective two-faced immune response utilizing multiple arms of resistance to influenza virus infection. Mip1α did presented itself as the best overall performer with the H7 antigen. This is in somewhat contradiction to previous data with the H1 (PR8) antigen, where T cell depleted mice immunized with MHCII targeted antigen were protected, while T cell depletion abrogated protection in mice immunized with Mip1α targeted antigen [209]. This highlights the fact that the immune response and the targeting effect is very antigen dependent and can differ even with very small variations in the antigen [172, 209] (Paper I).

Targeting of antigen to MHCII molecules is intuitive given their central position in B cell
responses, and has been extensively studied as targets on APCs [172, 175, 177, 188, 269]. MHCII-targeted H7 vaccines induced high titers of antigen-specific antibodies the first weeks after immunization, and also demonstrated increased neutralization against a H7 pseudotyped virus and reduced lung pathology (Paper I). MHCII-targeted H1 vaccines demonstrated increased amounts of IL-4 secreting cells in draining LNs and higher levels of CXCL13 in plasma indicating an increased GC reaction as compared to the non-targeted control vaccine [270,271] (Paper II). A higher number of plasma cells in the bone marrow was also found when targeting H1 to MHCII molecules, as compared to the non-targeted control vaccine (Paper II). This is in correspondence with the increased avidity of the GC B cell population that was observed, as high affinity clones are actively selected to the plasma cell compartment [41]. The body can sustain only a certain number of plasma cells and thus assures that only B cells with high affinity and high neutralization capacity are formed to establish effective humoral immunity. An acceleration of this process induced by MHCII-targeting is of special interest in vaccine development, and particularly for establishing humoral immunity towards novel influenza viruses in a pandemic setting.

Importantly, we have now established direct evidence through the T-B cell collaboration assays (Paper II) that MHCII-targeting increases presentation of antigenic peptides on MHCII. B cells generally display poor passive uptake and presentation due to the restriction to BCR specific uptake of antigen [75–77]. After BCR mediated uptake of antigen B cells process and present antigen to antigen specific helper T cells in the GCs [272]. Targeting to MHCII could activate B cells to function as APCs regardless of the BCR. However, B cell activation and proliferation is dependent on antigen specificity (Paper II). The immune potentiating effect when targeting antigen to MHCII was also observed in immune deficient NSG mice as recipients for antigen specific T and B cells (Paper II). The identical results with antigen specific T- and B cells transferred to BALB/c mice (presence of host contributing APCs) and NSG mice (no host APCs) further indicate that B cells have an enhanced function as APCs in the presence of MHCII targeted antigen. It is, however, important to note that this observation does not exclude a role of DCs in the BALB/c experiments. The enhanced B cell response observed when targeting antigen to MCHII in NSG mice might be a result of increased uptake and presentation of peptides on MCHII due to two possible modes of ligation via the BCR and MHCII molecules, possibly due to cross linking of BCR and MHCII on a single B cell (Fig.8A). Another, non-exclusive mechanism might be synapse formation between two B cells, equivalent to an APC-B cell synapse (Fig.8B).

Antibody responses are triggered when BCRs bind antigen on the surface of APCs such as DCs [31,273] or subcapsular sinus macrophages [24,26,30]. B cells are most sensitive
**Figure 8:** Illustration of the MHCII targeted vaccine and possible interactions with antigen specific B cells A) MHCII targeted antigen can ligate and cross link BCR, MHCII, and BCR-MHCII on a single B cell inducing uptake, processing and presentation of antigen peptide to CD4+ T cells. B) Homodimeric vaccine protein bound to MHCII molecules on APC can cross link BCRs on an antigen specific B cells in a B cell-APC synapse. Partly adapted from Fredriksen et al. [213].

To membrane bound antigen and acquire antigen by forming an immunological synapse in these areas of the LN [274,275]. Formation of immune complexes will increase the sensitivity of B cells several hundred fold, as these can associate antigen with membrane bound complement or Fc receptors [276–279]. The increased immunogenicity of immune complexes could be mimicked by APC-targeting of antigen, as relevant APCs could display antigen bound to their membrane via the targeted receptor (Fig. 8B) [213]. The bivalent display of antigen bound to receptors on APCs can then enhance antigen sampling and formation of the BCR activation cluster, SMAC. Increased affinity maturation might also be promoted as antigen interactions between B cells and follicular DCs are essential in the affinity maturation process [280–282]. Injection of protein or DNA vaccination with an APC-targeted protein could thus increase B-cell interaction with APC-displayed vaccine protein similar to the way B cells sense immune complexes, thus increasing immunogenicity and speed of the immune response.

Following DNA vaccination, targeting of antigen to MHCII has been shown to result in a lower systemic protein concentration as compared to a non-targeted control vaccine [177]. This indicates that the targeting unit might facilitate retention of antigen in lymphatic tissues. Both B cell proliferation and hypermutation is directly proportional to the amount of antigen capture by B cells in the follicles [283]. MHCII targeted antigen can bind subcapsular sinus macrophages that are particularly efficient in transporting intact protein.
into the follicles [24]. Vaccination with MHCII targeted antigen likely optimizes antigen retention in the draining LNs facilitating an enhanced GC reaction.

Future perspectives

Highly pathogenic avian influenza

HPAIV H5 and H7 strains constitute pandemic threats. A key problem and bottleneck in the pandemic vaccine preparations is to identify the appropriate time to switch from production of seasonal influenza vaccines to prioritize a pandemic vaccine. Pandemic DNA vaccines can essentially eliminate this bottleneck and enable a fast track production line and strain switch within the time necessary to start vaccination and quench a pandemic outbreak. Lists of candidate strains for H5 and H7 avian influenza are published and continually updated by the WHO [284, 285]. APC-targeted HA antigens from these potential pandemic strains need to be characterized in mice and developed for clinical phase I trials. The present thesis includes the basic work in mice for such an endeavor, and the next step would be to prepare the experiments necessary for completing a clinical dossier. MHCII-targeted DNA vaccines against H1N1 influenza have previously been tested in larger animals and non-human primates [175] (Grødeland et al., unpublished.) with encouraging results. Thus, there exists a pipeline for moving forward with development of vaccines for pandemic preparedness and phase I clinical trials in humans with H5 and H7 antigens are currently planned to be performed in 2019.

Other potential pandemics and epidemics

WHO pandemic and epidemic preparedness program lists a range of high priority viral pathogens that requires close attention in case of emergence. Among these diseases are avian influenza, ebola virus disease, zika virus and viral haemorrhagic fevers. Because many of these pathogens constitute a pandemic threat, vaccines for pandemic preparedness should be prioritized. APC-targeted DNA vaccines with immunogens from these pathogens should be investigated in pre-clinical studies. Because of the polarization of immune responses offered by the nature of the APC targeting unit (Paper II and III), the APC-targeted DNA vaccines can likely function as vaccines against a wide range of pathogens.
WHO presents guidelines for pandemic influenza vaccines and defines a “Pandemic Influenza preparedness Framework”. According to these guidelines, approval of an influenza vaccine requires clinical data from at least two strains of influenza, and can then be “fast tracked” to be antigenically matched to an emerging influenza strain. Thus, vaccine development can be better prepared against a novel influenza strains at the start of the pandemic. This type of approval would represent the ultimate goal for the influenza vaccines presented in this thesis.

Coalition for Epidemic Preparedness Innovations (CEPI) represents a mission to establish a new model for funding of vaccine development for preparedness against threatening pandemics and epidemics. Because infectious diseases are a global problem, and vaccination is highly cost effective and efficacious in minimizing outbreak and spread, there is a call for a collective effort to develop vaccines for a global solution.

**Induction of broadly neutralizing antibodies**

Broadly neutralizing antibodies (bnAbs) have been identified against influenza HA [146, 147] and HIV envelope protein [286, 287]. These antibodies target conserved epitopes in the surface proteins and can recognize a wide range of viral variants. BnAbs are induced by natural infections, but strong reactions to highly variable immunodominant epitopes lead to extremely low titers of bnAbs. Additionally, bnAbs are most prevalent in patients after long periods of continuous exposure such as in the elderly after many seasons of influenza virus exposure [288, 289] or patients chronically infected with HIV [286]. High boosting rates and multiple CDR mutations are associated with the development of bnAbs making them a challenge to induce by vaccination [290]. Due to the specific B cell activation elicited by MHCII targeting (Paper II), protein or DNA based vaccines aiming for specific induction of the precursors mediating bnAbs and affinity maturation against those epitopes can be realized. With the flexibility of DNA vaccines, sophisticated prime-boost immunogens [291,292] can be investigated in combination with MHCII targeting to identify an efficient method to raise bnAbs by vaccination.

**HA probes for identification of broadly reactive GC B cells**

Detection of broadly neutralizing B cells usually involves creating hybridomas or isolating single plasma cells to investigate the properties of the monoclonal antibodies in subsequent assays. Antigen specific detection of B cells in flow cytometry can identify the B
cell clones induced by vaccination (Paper II and Paper III). By using multiple HAs with separate fluorophores, either detected by specific tags and mAbs or recombinantly coupled to fluorescent proteins, B cells with broad reactivity can be identified by multicolor flow cytometry. This would allow an efficient method in screening HA reactivity during immunization aiming to induce broadly neutralizing antibodies.

**Polarization by targeting units**

Targeting of antigen to CCR 1, 3, and 5 or MHCII (Paper I and II) effectively targets antigen to a wide range of cells. The skewing of the immune response is clear with these targeting units [209], but the underlying mechanisms behind the polarization and the cells involved are not characterized. Because the CCR 1, 3, and 5, or MHCII targeted immunogen can interact with many immune cells, the specific interactions between T cells and the APC are not easily identified in an in vivo system. Targeting Xcr1 with the chemokine Xcl1 induces a strong Th1 type response [184], while targeting CD11c induces a Th2 response [211, 293] (Braathen et al. un-published). These two receptors are exclusively expressed on conventional DC type 1. Detailed studies of how polarization is initiated during the first phases after immunization are possible. T cells of the Th1 or Th2 type can be identified by expression of the master regulators T-bet and Gata3, respectively. Furthermore, T_{FH} express Bcl6. T cells recognizing MHCI or MHCII restricted ovalbumin peptides (OT-I and OT-II cells, respectively) can be utilized in a detailed study of polarization of immune responses by targeting DCs.

**Antigen and lymphocyte dynamics**

Previous data indicate that targeting antigen to MHCII retains immunogen in lymphatic tissue, compared to non-targeted control, after DNA vaccination. It is, however, not known in detail how the immunogen is transported to draining LNs and which cells are involved in presentation of antigen to B cells. Upon secretion of vaccine protein in the skin or muscle, protein might drain passively to LNs or be actively transported by migratory DCs. When antigen enters the paracortex, follicular DCs, macrophages or even B cells might be key in interaction with antigen specific B cells for initiation of the GC response. The dimeric APC-targeted vaccine format is effectively produced with mCherry as antigenic unit [244]. Injection of mCherry targeted to APCs in combination with non-targeted control could give a better insight to antigen trafficking and retention
when targeted to receptors on APCs. These types of experiments could be extended to injection of APC-targeted mCherry and scFv\textsuperscript{315} heterodimeric vaccine proteins [294] in the presence of antigen specific B cells in the LNs. Immunostaining of LN sections could then allow visualization of the interaction between follicular DCs, APC-targeted antigen, and B cells in the GC. Ideally, experiments with anti-mCherry knock in BCR mice and APC targeted mCherry antigen could open possibilities for in depth analysis of antigen and lymphocyte dynamics after immunization with APC targeted antigen. This type of experiment might shine light on the interaction between B cells and DCs in the germinal center reaction, and if the increased B cell activation is induced by APCs facilitating a B cell-APC synapse.

**mRNA vaccines**

Efficacy of mRNA vaccines has been demonstrated in influenza A challenge experiments in mice and larger animals [295]. However, mammalian cells possess components of innate immunity as a defense against viral RNA and foreign RNA is quickly recognized and broken down [296, 297]. Furthermore, cellular compartmentalization prevents mRNA from passively entering the cytosol and mRNA delivery thus requires sophisticated delivery methods. Sequence optimization, incorporation of untranslated regions and a long poly A tail can stabilize mRNA [298–300]. Additionally, using chemically modified nucleotides evade ligation of PRRs and host innate immune responses and increase in vivo stability resulting in increased translation [301–303]. All these measures can increase translation efficiency of therapeutic mRNA constructs, but will also lower immunostimulatory effects. These properties of mRNA can be utilized to create a self adjuvanted mRNA vaccine combining translation efficiency and immune stimulating activity [304]. Preliminary studies have shown that mRNA vaccines encoding H1 (PR8) targeted to MHCII can be expressed in vitro, but gave weak responses in vivo, even after boost and high doses of mRNA (Andersen et al., unpublished). Further experiments with mRNA vaccines will aim at optimizing delivery methods to reach protective antibody levels in serum after a single immunization in mice.
Appendix

Abbreviations

ADCC  Antibody Dependent Cell Cytotoxicity
AID   Activation-induced cytidine deaminase
APC   Antigen Presenting cell
BCR   B-cell Receptor
bnAb  Broadly neutralizing antibody
CDC   Centers for Disease Control and Prevention
CDR   Complementarity-Determining Region
CEPI  Coalition for Epidemic Preparedness Innovations
CTL   Cytotoxic T-lymphocytes
DC    Dendritic Cell
ELISA Enzyme-Linked Immunosorbent Assay
ELISPOT Enzyme-Linked Immunosorbent Spot
ER    Endoplasmic Reticulum
FcR   Fc Receptor
HA    Hemagglutinin
HEV   High Endothelial Venule
HI    Hemagglutinin Inhibition
HPAIV High Pathogenic Avian Influenza Virus
IFN-γ Interferon gamma
Ig    Immunoglobulin
IL-4  Interleukin 4
ITAM  Immunoreceptor Tyrosine-based Activation Motif
LN    Lymph node
MBCS  Multibasic Cleavage Site
MHC   Major Histocompatibility Complex
NA    Neuraminidase
NP    Nucleoprotein
PAMP  Pathogen Associated Molecular Pattern
PRR   Pattern Recognizing Receptor
RAGs  Recombination-activating Genes
SA    Sialic Acid
scFv  Single Chain Variable Fragment
SHM   Somatic Hyper Mutation
SMAC  Supramolecular Activation Cluster
T<sub>FH</sub> Follicular T helper cells
TCR   T-cell Receptor
Treg  Regulatory T cells
WHO   World Health Organization
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Publications
A DNA vaccine that targets hemagglutinin to antigen presenting cells protects mice against H7 influenza

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Running Head: APC-targeted DNA vaccine protects against H7 influenza

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Abstract:
Zoonotic influenza H7 viral infections have a case fatality rate of about 40%. Currently no or limited human to human spread has occurred, but we may be facing a severe pandemic threat if the virus acquires the ability to transmit between humans. Novel vaccines that can be rapidly produced for global distribution are urgently needed, and DNA vaccines may be the only type of vaccine that allows for the speed necessary to quench an emerging pandemic. Here, we have constructed DNA vaccines encoding the hemagglutinin (HA) from influenza A/chicken/Italy/13474/99 (H7N1). In order to increase the efficacy of DNA vaccination, HA was targeted to either major histocompatibility complex (MHC) class II molecules or chemokine receptors 1, 3 and 5 (CCR1/3/5) that are expressed on antigen presenting cells (APC). A single DNA vaccination with APC-targeted HA significantly increased antibody levels in sera, as compared to non-targeted control vaccines. The antibodies were confirmed neutralizing in an H7 pseudotype-based neutralization assay. Furthermore, the APC-targeted vaccines increased the levels of antigen-specific cytotoxic T-cells, and a single DNA vaccination could confer protection against a lethal challenge with influenza A/turkey/Italy/3889/1999 (H7N1) in mice. In conclusion, we have developed a vaccine that rapidly could contribute protection against a pandemic threat from avian influenza.
Importance:

Highly pathogenic avian influenza H7 constitute a pandemic threat that can cause severe illness and death in infected individuals. Vaccination is the main method of prophylaxis against influenza, but current vaccine strategies fall short in a pandemic situation due to a prolonged production time and insufficient production capabilities. In contrast, a DNA vaccine can be rapidly produced and deployed to prevent the potential escalation of a highly pathogenic influenza pandemic. We here demonstrate that a single DNA delivery of hemagglutinin from an H7 influenza could mediate full protection against a lethal challenge with H7N1 influenza in mice. Vaccine efficacy was contingent on targeting of the secreted vaccine protein to antigen presenting cells.
Introduction

Highly pathogenic avian influenza viruses (HPAIV) represent a potential pandemic threat. As of June, 2017, WHO has reported a total of 1533 laboratory-confirmed cases of human infection with avian influenza H7N9, with a mortality rate of nearly 40% (1). A majority of these cases arose from zoonotic transmissions at the human-animal interface, with limited human to human transmission. Viruses isolated from human cases, perhaps including secondary cases of human transmissions, show only a few accumulated mutations in surface glycoproteins (2). Thus, it is difficult to predict the antigenic determinants of transmissibility that are needed to break the zoonotic barrier, and also how these would influence the viral pathogenicity in humans (3).

However, the human population is presently serologically naive towards H7 influenza, therefore the potential acquisition of mutations enabling efficient human to human transmission could have a devastating effect.

Conventional vaccine design relies on an extensive surveillance system to determine which influenza strains will be included in the next season’s influenza vaccine. The 2009 pandemic demonstrated that the development and vaccine production process could be completed in about six months, which represents a best case scenario (4).

Both the 2009 H1N1 pandemic and the 2013 HPAIV H7N9 emergence in China (5) demonstrate that it is difficult to predict which influenza strain will cause the next pandemic, and that conventional influenza vaccines are not sufficient in the face of a pandemic outbreak. Novel vaccine formats that can rapidly be produced and quickly induce an immune response upon a novel pandemic threat are urgently needed (6-8).

The multifaceted pathogenicity of HPAIV is maintained by two major determinants.
Firstly, the hemagglutinin (HA) in HAPIV has a receptor binding preference for α2,3-linked sialic acid that is abundant on the gut epithelia of aquatic birds (9). In humans, α2,3- and α2,6-linked sialic acid receptors dominate in the lower and upper respiratory tract, respectively. Efficient human to human transmission of influenza virus is dependent on viral replication in the upper respiratory tract (10). The viral preference for α2,3-linked sialic acid receptors thus represents a natural barrier for zoonotic and human-to-human transmission with HPAIV (11). However, certain H7 isolates have been demonstrated to bind both sialic acid receptors (12-14), forming a breach in the zoonotic barrier (15). Secondly, HA in HPAIV have acquired a multibasic cleavage site (MBCS) (16-19). HA cleavage is necessary for influenza infectivity, and where seasonal influenza HAs are only cleaved by tissue restricted proteases, HAPIV HAs can be cleaved by ubiquitous cellular proteases. Thus, the potential pathogenicity of an HPAIV is enhanced since the virus evades tissue restricted replication (20-23).

DNA vaccines can be rapidly produced, but are typically hampered by reduced immunogenicity. Previously, we have demonstrated that a single DNA vaccination with HA from influenza H1N1 targeted to MHCII molecules on APCs confers sterilizing immunity against influenza challenge in mice (24, 25). Furthermore, translation into larger animals confirmed the increased immunogenicity after APC-targeting of antigen (26). In addition to MHCII targeting, chemokines are attractive targeting units due to chemotactic recruitment of, among others, DCs, macrophages and NK-cells, and can channel recruitment of internalized antigen to MHCI and MHCII presentation pathways (27-31). Here, we have extended these experiments to vaccination against HPAIV H7. Following targeting of HA from
A/chicken/Italy/13474/1999 (H7N1) or A/turkey/Italy/3889/1999 (H7N1) to MHCII molecules or chemokine receptor 1, 3, and 5 (CCR1/3/5) expressed on APC, we here demonstrate that a single DNA vaccination can confer protection against a lethal H7N1 challenge in mice.
Results

Construction and characterization of APC-targeted influenza vaccines

Previously, we have demonstrated that a single DNA immunization with APC-targeted HA from H1N1 influenzas provided protection against a lethal challenge in mice (23, 24). A key feature of the vaccine was bivalent display of antigens, that were linked, via a dimerization unit containing the hinge region and C\text{H}3 from human IgG3, to targeting units specific for receptors on APCs (Fig. 1A)(28, 32). Here, we have inserted HA from A/chicken/Italy/13474/99 (H7N1) (aa19-536) into the same vaccine format, and targeted HA towards either chemokine receptors 1, 3, and 5 (CCR1/3/5), or MHC class II molecules (Fig. 1A). For targeting of HA to MHC class II molecules we used a single chain variable fragment (scFv) that was specific for I-E\text{d} (denoted \alpha\text{MHCII-H7}), whereas the chemokine Mip1\alpha was used for targeting of HA to CCR1/3/5 (denoted Mip1\alpha-H7). As non-targeted controls, we constructed a vaccine where the APC-specific targeting unit was replaced with a scFv against the hapten Nip (denoted \alpha\text{Nip-H7}), and a plasmid encoding only HA (denoted H7). In some experiments, we also used the previously described MHCII-targeted vaccine with HA from influenza A/Puerto Rico/8/34 (H1N1)(24) to control for antigen specificity (denoted \alpha\text{MHCII-H1}).

Supernatants from 293E cells transiently transfected with the different vaccine plasmids confirmed the appropriate size (Fig.1B) and \textit{in vitro} expression of the secreted vaccine proteins (Fig.1C). The non-targeted control, \alpha\text{Nip-H7}, exhibited an about two-fold higher protein expression level than the other vaccines, potentially providing a concentration dependent benefit for the \textit{in vivo} DNA vaccinations. In order to verify efficient binding of the vaccine proteins to APC, we assayed their
binding profiles to splenocytes. (Fig.1D). Vaccines targeting MCHII molecules ligated B-cells, macrophages and conventional DC 1 and 2, while CCR1/3/5-targeted vaccines ligated macrophages and conventional DC 1. As expected, non-targeted control failed to ligate any of the cell types investigated, and T-cells were not bound by either of the constructs.

An intact multibasic cleavage site increases antibody responses

Conventional production of vaccines against highly pathogenic avian influenza viruses depends on removal of the MBCS in order to prevent disease and potential lethality embryonated hens’ eggs (4, 33). In contrast, this is not a problem for the synthetic production of DNA vaccines. Therefore, we wanted to compare the immunogenicities of APC-targeted H7 antigens with the intact or deleted MBCS sequences, and cloned an HA with a deleted MBCS into the MHCII-targeted vaccine format described above. The vaccine displaying the deleted MBCS sequence was denoted αMHCII-H7Δ (Fig.2A).

The correct size and expression of αMHCII-H7Δ was confirmed (Fig. 2C-D). As endogenous proteases in 293E cells will cleave H7 with intact MBCS(22), we observed bands corresponding to cleavage of HA0 into HA1 (about 115 kDa, including the targeting domain and dimerization unit) and HA2 (about 35kDa) for αMHCII-H7, but not for αMHCII-H7Δ (Fig. 2B-C). The in vitro expression levels of αMHCII-H7Δ were slightly higher than that of αMHCII-H7 (Fig. 2D). Nevertheless, in vivo DNA vaccination of mice showed that αMHCII-H7 induced significantly higher IgG titers as compared to αMHCII-H7Δ (Fig. 2E). The difference was significant already one week post vaccination, and antibody levels remained higher at
least four weeks post vaccination. Taken together, the data suggest that H7 with an intact MBCS could offer an immune-potentiating effect that is maintained when targeting of HA to MHC class II molecules in mice. Thus, further experiments were performed with αMHCII-H7.

Rapidly increased IgG levels after MHCII-targeted vaccination

As rapid induction of protection is crucial for pandemic prevention, mice were DNA vaccinated only once and antibody responses in serum assessed for five weeks post vaccination. Mice were immunized with plasmids targeting H7 towards either CCR1/3/5 or MHCII molecules, or with non-targeted control vaccines (αNip-H7 and H7). In addition, a group was vaccinated with αMHCII-H1 to control for specificity (Fig. 3A). To generalize the effect of APC targeting, parallel experiments were performed in BALB/c mice and the CB6F1 hybrid strain. Results demonstrated that IgG responses were significantly elevated already the first weeks after vaccination with αMHCII-H7 in both BALB/c (Fig. 3B) and CB6F1 mice (Fig. 3C). The antibody responses after vaccination with Mip1α-H7 were only significantly increased above that of the non-targeted controls at week 4 post vaccination for both mouse strains. As expected, αMHCII-PR8 did not induce any antibodies that cross-reacted with influenza H7. The same trends were observed when the vaccines were delivered i.m. in BALB/c (data not shown).

In order to assess the neutralizing antibody response, sera from week 2 and 4 post vaccination in BALB/c were examined in a pseudotype-based neutralization assay against A/FPV/Rostock/1934(H7N1) HA and NA pseudotype virus. Results demonstrated that a single vaccination with αMHCII-H7 significantly increased IC₅₀
levels in sera at both 2 and 4 weeks post vaccination, as compared to the non-targeted control vaccines (αNIP-H7 and H7) (Fig.3D). Furthermore, significantly enhanced IC₅₀ levels were observed in sera collected 4 weeks after vaccination with Mip1α-H7, as compared to the non-targeted controls. The results for both αMHCII-H7 and Mip1α-H7 corresponded with the increases that were observed in ELISA (p<0.05, Spearman correlation IC₅₀ and serum titers) (Fig. 3B and D). When extending the analyses to examinations of IC₉₀ titers, vaccination with αMHCII-H7 significantly increased the levels of protective antibodies, as compared to non-targeted controls. Taken together, the data demonstrated that αMHCII-H7 is superior at induction of neutralizing antibodies, as compared to Mip1α-H7 and non-targeted controls.

Vaccine induced production of IFNγ-secreting cells and cytotoxic T lymphocytes

While antibodies may block an influenza infection, T cells can clear already infected cells. Thus, the different DNA vaccines were assessed for their ability to induce sustained IFNγ secreting cells and cytotoxic T lymphocytes (CTL) after immunization. Splenocytes were harvested 8 weeks post vaccination, and re-stimulated with recombinant HA from H7N9 influenza. Results demonstrated that mice receiving either αMHCII-H7 or Mip1α-H7 induced significantly higher levels of IFNγ secreting cells, as compared to the non-targeted controls (Fig. 4A). The enhanced induction was specific for H7, as re-stimulation with HA from other influenza A subtypes failed to raise IFNγ production. As an additional control, αMHCII-H1 increased the levels of IFNγ-secreting cells only after stimulation with HA from H1N1 influenza (Fig. 4B).
To further address if the increase in IFNγ secreting cells could be interpreted as increased CTL activity *in vivo*, we retrovirally transduced an A20 mouse B lymphoma cell line to express cytosolic H7 and GFP, or mCherry as a control. At week 5 post vaccination the cell lines were injected into BALB/c mice, and the ratio between GFP and mCherry positive cells investigated 16h later by flow cytometry. The killing ratios were calculated by comparing the remaining GFP population to the control population expressing mCherry (Fig. 4C). Both APC-targeted vaccines induced significant CTL responses, and in correspondence with the ELISPOT data, CCR1/3/5-targeting induced a higher T-cell response as compared to the MHCII-targeted vaccine. In sum, the data demonstrated that targeting of HA to APCs significantly increased the vaccine induced T-cell responses, and that they can participate in protection against an influenza infection.

*A single APC-targeted DNA vaccination can protect mice from a lethal influenza challenge*

In order to examine if the APC-targeted vaccines could rapidly confer protection against influenza, mice were DNA immunized once i.d. and then challenged with a lethal dose of influenza A/turkey/Italy/3889/1999 (H7N1) five weeks after vaccination. Mice that received the APC-targeted vaccines showed significantly less morbidity as compared to the non-targeted controls, and both BALB/c (Fig.5A) and CB6F1 mice (Fig.5B) were fully protected against influenza. In contrast, mice that received saline or non-targeted controls rapidly lost weight, and 40 to 60% of mice receiving the non-targeted control vaccines had to be euthanized within 8 days post challenge.
The influenza strain used for challenge has some sequence differences as compared to the vaccine strain. In order to examine protection in a homologous challenge model, we also immunized mice with HA from A/turkey/Italy/3889/1999 (H7N1) and challenged with this strain 5 weeks later (Fig.5C). Mice receiving APC-targeted vaccines were fully protected against the lethal influenza challenge, in contrast to the non-targeted control vaccines. For the heterologous challenge experiments above (Fig.5A-B), APC-targeting of HA significantly improved protection against the influenza challenge when compared to the non-targeted control vaccines, but we also observed an increased weight loss in mice receiving MHCII-targeted HA as compared to the group vaccinated with Mip1a-H7. Here, we observed no significant difference in weight loss after MHCII- and CCR1/3/5-targeted vaccination in the homologous challenge (Fig.5C).

In order to further examine the differences in protection following vaccination with MHCII- and CCR1/3/5-targeted HA, we set up an experiment examining lung pathology after viral challenge in BALB/c mice. Thus, mice were immunized with HA from A/chicken/Italy/13474/1999 (H7N1) and challenged five weeks later with A/turkey/Italy/3889/1999 (H7N1). At day 7 post challenge, lungs were harvested, sectioned and H&E stained. Lungs harvested from control mice receiving NaCl and non-targeted control vaccines displayed histiocytic alveolitis, interstitial pneumonia and edema. In contrast, mice receiving APC targeted vaccines had healthy lungs with only minor lung pathology (Fig.5D). Further, we observed that lungs collected from mice vaccinated with Mip1α-H7 had more cellular infiltration and lung pathology, as compared to mice vaccinated with αMHCII-H7.
Targeted vaccines induce T-cell responses that contribute to protection against viral challenge

In order to investigate whether the main mediator of protection was antibodies or T cells, immunized mice were depleted for CD4+ and CD8+ T cells just prior to a lethal influenza challenge. The depletion was maintained by injections of CD4 and CD8 depleting antibodies every other day, and was determined to have an efficacy level of >99% (data not shown). T-cell depleted mice showed no significant increase in mortality compared to mice receiving isotype matched control antibodies. This demonstrated that the induced antibodies were protective. However, recovery after infection was significantly impaired in T-cell depleted mice, as shown by the significant differences in morbidity that were observed during the second week after infection. The experiment highlights the importance of T cells in clearing a heterologous influenza infection. In conclusion, the APC-targeted vaccination induced both antibodies and T-cells that contribute to protection against influenza.
Discussion

In the event of a pandemic outbreak of HPAIV, a vaccine platform capable of launching a quick production and response is crucial. It is essential to rapidly induce protective immunity in the population to limit viral spread and disease. We have here demonstrated that DNA vaccines targeting HA from HPAIV H7 to APCs in mice represent a potential vaccine candidate for induction of rapid immunity. Importantly, the APC-targeted DNA vaccines could mediate protection in mice that were challenged with a lethal dose of influenza H7N1 virus only five weeks after vaccination.

We have here used HA from either A/turkey/Italy/3889/1999 (H7N1) or A/chicken/Italy/13474/1999 (H7N1) for vaccination, and observed that both vaccines can confer protection against a viral challenge with A/turkey/Italy/3889/1999 (H7N1). Thus, the induced immune responses can to some extent confer heterologous protection against different H7 strains. While this cannot at present be generalized to more H7 strains, we also found that vaccination with HA from A/chicken/Italy/13474/1999 (H7N1) raised neutralizing antibodies against A/FPV/Rostock/1934 (H7N1) in a pseudotype-based neutralization assay.

In order to further investigate the mechanisms behind protection, mice vaccinated with Mip1α-H7 or αMHCII-H7 were injected with depleting antibodies against CD4+ and CD8+ T cells, or isotype matched control antibodies. Following challenge with the H7N1 virus, no significant differences were observed in survival between the T cell depleted mice and isotype treated mice. While this indicated that cross-reactive antibodies contributed to the protection, T-cell depleted mice showed a significant
increase in morbidity and delayed recovery post challenge. Presumably, the vaccine
induced neutralizing antibodies that could recognize the heterologous virus were
below the limiting threshold necessary to mediate complete sterile protection at the
given dose. In this situation, a recall of a neutralizing memory B-cell repertoire during
the first days of infection likely inhibited ongoing viral infections, but the lack of viral
clearance in already infected cells led to some disease manifestations. This
observation held true both for mice vaccinated with αMHCII-H7 and Mip1α-H7.

Previously, we have demonstrated that targeting of antigen to MHCII molecules is
particularly efficient for induction of antibodies, whereas targeting of antigen to
CCR1/3/5 more efficiently will induce T-cell responses(25). Following vaccination
with A/chicken/Italy/13474/1999 (H7N1) and challenge with
A/turkey/Italy/3889/1999 (H7N1), we observed that mice vaccinated with Mip1α-H7
had a significantly reduced weight loss as compared to αMHCII-H7. In contrast, we
found no significant difference in weight loss between mice vaccinated with αMHCII-
H7 or Mip1α-H7 when HA from the homologous strain was used. T cells have an
increased potential for mediating cross protection within variations of antigenic
drift(34), as T-cell epitopes are more conserved across different subtypes(35). It is
therefore likely that increased numbers of IFNγ secreting T cells and the slight
increased CTL response after Mip1α-H7 vaccination, as compared to αMHCII-H7,
can account for the difference in weight loss observed after heterologous challenge.
Furthermore, the antibody response after Mip1α-targeted vaccine delivery is
dominated by IgG2a(25). Non-neutralizing H7 specific IgG2a antibodies have also
been demonstrated to be protective in mice(36, 37). Antigen specific antibodies of the
IgG2a isotype efficiently induce Fc-receptor-mediated effector functions such as
antibody-dependent cell-mediated cytotoxicity (ADCC)(38, 39), complement dependent cytotoxicity(40, 41), or antibody dependent cell phagocytosis(42, 43). Additionally, ADCC triggering antibodies have been shown to be cross reactive(44), and might be of particular importance in a pandemic setting(44, 45). In summary, both MHCII and CCR1/3/5 targeting vaccines induced humoral and cellular responses that contributed to the mode of the observed protection.

Targeting of H7 to APCs consistently increased immune responses, as compared to non-targeted controls. Of note, a direct ligation between antigen and targeting unit has been found a prerequisite for efficient induction of immunity(46). It is possible that binding of an APC-targeted vaccine to an APC will improve access to antigenic epitopes, and as such promote more efficient binding to B-cell receptors (BCR). A stronger and more specific ligation of DCs or B-cells might drive an accelerated formation of germinal centers with subsequent isotype switching and affinity maturation. Furthermore, the APC-targeted vaccine could bridge APCs and B-cells in a synapse that promotes efficient and mutual activation(47, 48). Furthermore, a synapse formation between an APC and B-cell could lead to B-cells sampling antigen from the top, i.e. head region of HA. This might lead to selection B-cells recognizing more neutralizing epitopes in HA resulting in an antibody repertoire with highly neutralizing capacity as observed with the MHCII targeted vaccine.

Our data have clearly demonstrated the benefit of targeting antigen to APCs, particularly when the aim is to induce rapid immune responses to limit the outbreak of pandemic influenza. Previous studies have demonstrated that the immune potentiating effect when targeting to APCs is particularly prominent for weak antigens(28, 32). In
contrast, HA from HPAIV H7 is immunogenic in mice, and vaccination with the H7
antigen alone was able to induce modest IgG titers over time. It is possible that avian
HAs have increased immunogenicity as compared to human HAs due to differences
in glycosylation patterns that would stimulate production of immunostimulatory
agents in DCs(49). Additionally, inactivated influenza viruses with an α2,3
preference for sialic acid receptors have been shown to induce higher levels of
proinflammatory cytokines in human DCs(18). Thus, HA from HPAIV might trigger
an innate immune response that translates into activation of adaptive immunity in
mice. However, the antibody levels observed after immunization with non-targeted
controls developed significantly slower as compared to the APC targeted vaccines.
Furthermore, the non-targeted controls do not enhance immune responses to the same
extent as the APC-targeted vaccines, and, most importantly, only APC targeted
vaccines could induce protective immunity, after a single vaccination, towards a lethal
challenge with influenza virus.

When using conventional vaccine manufacture for selected avian influenza strains,
removal of the MBCS and exchange of certain internal genes is required(4). However,
a removal of the MBCS can render the virus low pathogenic and poorly
immunogenic, compromising vaccine efficacy with obvious implications for vaccine
design against pandemic outbreaks(17, 50). Here, we also found that deletion of the
MBCS was associated with reduced immunogenicity, which again highlights a benefit
of using DNA based vaccines. Ubiquitous proteases will cleave H7 in the vaccine
molecules in vivo. Cleavage of HA in the APC targeted vaccine molecule results in
efficient presentation of the native head region in HA1. Vaccines expressed with
uncleaved HA0 might lack some of the essential structural epitopes in HA1 or the
HA1/HA2 interface. The majority of protective antibodies bind to the globular head
domain of HA. Thus, the cleaved H7 format could more efficiently present epitopes
from native HA1, potentially resulting in a more stringent selection of B-cells with
neutralizing capacity. This effect is probably tissue and species dependent(23, 51), but
is a general benefit from DNA vaccines.

We have here demonstrated that a single DNA vaccination with APC-targeted H7 can
confer protection against H7 influenza in mice. No other vaccine format can at
present compete with the speed of production and deployment that is possible for
DNA vaccines. For prophylactic mass vaccinations, DNA vaccines can be delivered
without adjuvant to the dermis by non-invasive needle-free jet delivery systems(26,
52). Ideally, the DNA vaccine should be tailored to precisely match an emerging
pandemic strain, which is also a feasible strategy given the short time in which DNA
vaccines can be mass produced. The rapid induction of protective antibodies observed
when targeting H7 to MHCII molecules highlights this receptor as particularly
interesting for construction of pandemic DNA vaccines. For broader protection, it
could be beneficial to target the H7 to chemokine receptors. In summary, we have
here demonstrated that DNA delivery of an APC-targeted vaccine could greatly aid
the control of an unexpected pandemic threat.
Materials and methods

Molecular cloning

The HA genes from A/chicken/Italy/13474/1999 (H7N1) (aa19-536) and A/turkey/Italy/3889/1999 (H7N1) (aa19-536) were ordered with flanking SfiI-sites (GenScript, Piscataway, NJ, USA), and cloned into the previously described hCMV-based pLNOH2 vaccine vectors equipped with targeting units consisting of either a single chain variable fragment (scFv) specific for MHCII (IEd)(32), a scFv specific for the hapten 4-hydroxy-3-iodo-5-nitrophenylacetic acid (NIP) (non-targeted control)(32), or the macrophage inflammatory protein 1-alpha (Mip-1α)(25, 28). In another vaccine construct, the multibasic cleavage site (aa340-343) was removed from HA (H7Δ), and the gene cloned into a vaccine vector with an MHCII-specific scFv. Additionally, a vaccine construct encoding only HA was constructed by introduction of an upstream BsmI restriction site with primers: 5’-

GGTGTGCATTCCGCGCCTCGGTG and

3’GTGGATCTCTCAGTGACGGAGCCGGC (BsmI- and start of SfiI-sites are underlined).

Assessment of in vitro expressed vaccine proteins

An Influenza A H7N9 (A/Shanghai/1/2013) hemagglutinin ELISA Pair set (SEK40104, Sino Biological Inc., North Wales, PA, United States) was used. Briefly, ELISA plates (Costar 3590, Corning, NY, USA) were coated with 0.5μg/ml rabbit anti-HA mAb, blocked with 1% BSA, and incubated with supernatants from 293E cells transfected with 1μg vaccine plasmids and 2μg polyethylenimine (PEI). Vaccine proteins were detected with horseradish peroxidase (HRP) conjugated anti-HA pAb (1μg/ml) and 3,3’,5,5’-Tetramethylbenzidine TMB substrate (CL07, Merck Millipore,
San Diego, CA, USA). The reaction was stopped after 20min incubation with an equal volume of 0.5M H$_2$SO$_4$, and plates were read at 450nm with a Tecan reader (Tecan, Switzerland) using the Magellan v5.03 software.

Vaccine proteins were produced by transient transfection of 293E cells with PEI. Constructs containing the C$\beta_3$ domain were purified on a CaptureSelect FcXL Affinity column (194328005, Life Technologies, Naarden, The Netherlands).

Splenocytes were harvested from BALB/c and single cell suspensions were FcγR-blocked by incubation with 30% heat inactivated rat serum, and stained with purified vaccine proteins, CD3-VF450 (75-0032, TONBO biosciences, San Diego, CA, USA), CD19-FITC (35-0193, TONBO biosciences), CD11b-PerCP/Cy5.5 (65-0112, TONBO biosciences), CD11c-PE/Cy7 (117318, BioLegend, San Diego, CA, USA), F4/80-AF700 (123130, BioLegend), and CD64-APC (139306, BioLegend), followed by hCH3-PE (409304, BioLegend). Cells were analyzed on an Attune NxT flow cytometer (Thermo Fisher Scientific, Waltham, MA, USA) and FlowJo software.

**Western blot**

Vaccine plasmids were transiently transfected into 293E cells as described above. Supernatants were up-concentrated (VivaSpin 500, MWCO 10000, Goettingen, Germany) and denatured in SDS sample buffer at 95°C for 5min. The samples were then run on a Bolt™ 4-12% Bis-Tris Plus Gel (NW04122BOX, Novex, Carlsbad, CA, USA), blotted to a PVDF membrane (IB24001, iBlot® Transfer Stack, PVDF, Invitrogen, Kiryat Shmona, Israel), blocked in 2% skimmed milk, and detected with monoclonal (Fig.1) or polyclonal (Fig.2) rabbit αH7 (SEK40104, Sino Biological Inc.). Next, the membrane was incubated with polyclonal goat α-rabbit IgG conjugated to
ALP (A3687, Sigma-Aldrich), and developed with the BCIP®/NBT-Purple Liquid Substrate System for Membranes (B3679, Sigma-Aldrich).

**Mice and cell lines**

Cell work was performed with human embryonic kidney 293E cells purchased from the American Type Culture Collection (Manassas, VA, USA). Six to eight weeks old female BALB/c or CB6F1 (Janvier, le Genest-Saint-Isle, France) were used in all experiments. The animals were housed under minimal disease conditions, and all animal experiments were pre-approved by the Norwegian Animal Research Authority (NARA).

**Vaccination**

Mice were anesthetized [0.1mg/10g body weight with cocktail of: Zoletil Forte (250 mg/ml) (Virbac France), Rompun (20 mg/ml), (Bayer Animal Health GmbH), and Fentanyl (50 µg/ml) (Actavis, Germany)] by intraperitoneal (i.p.) injection. For intradermal (i.d.) delivery of vaccines, mice were shaved in the lower back region, and 12.5µg plasmids in a 25µl volume were injected at two sites (total DNA/mouse: 25µg) immediately followed by skin electroporation (EP) (DermaVax, Cellectis, Paris, France). For intramuscular (i.m.) delivery of vaccines, mice were shaved on each leg, and 6.25µg DNA injected in a 50µl volume into each quadriceps femoris (total DNA/mouse: 12.5µg). Immediately after injection, electrical pulses were applied at the injection site (Elgen, Inovio Biomedical Co., Blue Bell, PA, USA). All DNA vaccines were purified by Qiagen EndoFree Plasmid Mega Kit (12381, Qiagen, Hilden, Germany) and dissolved in sterile injection fluid (0.9%NaCl).
Serum ELISA

Blood was harvested by puncture of the saphenous vein, and sera isolated by centrifugation. ELISA plates (Costar 3590) were coated with 0.5µg/ml rec. HA [A/Shanghai/1/2013 (H7N9)] (40104-V08B, Sino Biological Inc.), blocked with 1%BSA, and incubated with serially diluted serum samples assayed individually (n=6-12/group). HA-specific antibodies were detected with alkaline phosphatase conjugated goat anti-mouse IgG (A1418, Sigma-Aldrich), developed with phosphatase substrate (P4744, Sigma Aldrich), and analysed as previously described. For all serum ELISAs, titers were determined as the last serum dilution with OD above background (mean absorbance from NaCl vaccinated mice added 5 times the standard error of the mean for the group).

Pseudotype-based neutralizing assay

Pseudotyped virus was prepared and quantified as previously reported(53). Briefly, 3x10^3 MDCK cells were seeded in each well of a 96-well culture plate (Corning, NY, USA), and incubated overnight at 37°C in 5% CO_2 and saturated humidity. Then, serially diluted serum samples, pooled by group, that had been pre-incubated with HA and NA pseudotypes from A/FPV/Rostock/1934 (H7N1) [2,000-200,000 relative luciferase activity (RLA)] at 37°C in 5% CO_2 and saturated humidity for 1h, were added to the cells for a 72h incubation at 37°C in 5% CO_2 and saturated humidity. RLA was measured by a BrightGlo Luciferase assay according to the manufacturer’s instructions (Promega, Madison, WI, USA). The percentage of inhibition was calculated by: (RLA in pseudotypes and medium control – RLA in pseudotypes and immune serum at a given dilution)/RLA in pseudotypes and medium control. The data
was fitted to a sigmoidal dose response curve using GraphPad Prism 6 software, and IC50 and IC90 were determined from those data sets.

**ELISPOT assay**

Mouse IFNγ ELISpot PLUS plates (3321-4APT, Mabtech, Nacka Strand, Sweden) were blocked with RPMI+10%FCS for 2h at 37°C in 5% CO₂. Mice spleens were harvested eight weeks post infection and single cell suspensions were prepared with the gentleMACSTM Dissociator (Milteny Biotec, Bergisch Gladbach, Germany), followed by incubation in ACT lysis buffer for 5min on ice. Next, 0.5x10⁶ cells were seeded per well, and cells stimulated with either medium (negative control), or ConA (1µg/ml, positive control), or 10µg/ml rec. HA from either A/Shanghai/1/2013(H7N9), or A/Puerto Rico/8/1934(H1N1), or A/Vietnam/1194/2004(H5N1) (40104-V08B, 11684-V08H, and 11062-V08H1, respectively, Sino Biologic Inc.). Plates were now incubated for 20h at 37°C in 5% CO₂, before incubation with the detection antibody (3321-4APT, Mabtech) and development with the BCIP®/NBT-Purple Liquid Substrate System for Membranes (B3679, Sigma-Aldrich). Plates were analyzed with the CTL-ImmunoSpot® analyzer (CTL, Shaker Heights, OH, USA).

**In vivo killing assay**

A20 cells expressing cytosolic H7 and GFP, and control cells expressing mCherry, were created by retroviral transduction followed by selection of high expressing cells by FACS. H7 GFP cells and mCherry cells (5x10⁶: 1:1) were injected i.v. into BALB/c mice, and the prevalence of GFP positive and mCherry positive cells in the spleen were analyzed 16h later in an Attune NxT flow cytometer (Thermo Fisher Scientific, Waltham, MA).
Scientific, Waltham, MA, USA) and with the FlowJo software. Killing ratios were calculated by defining the average ratio between the two cell lines in the NaCl group as 0% killing and finding no H7 GFP cells as 100% killing.

Viral challenge

Mice were anesthetized as described previously, and inoculated with 20xLD$_{50}$ mouse adapted A/turkey/Italy/3889/1999 (H7N1) in 10µl/nostril. Mice were monitored for weight loss, and mice that lost >20% of the original body weight were euthanized by cervical dislocation.

H&E staining of lung sections

Formalin fixed lungs were embedded in paraffin, sectioned, and stained with H&E. Sections were mounted using Dako Toluene-Free Mounting Medium (CS705, Dako, Santa Clara, CA, USA). Micrographs of tissue sections were collected using Nikon Eclipse Ti-S microscope (Nikon Corporation, Tokyo, Japan), and a 10x/0.30 objective.

T-cell depletion

Mice [n=10/n=8 (NaCl group)] were vaccinated once i.d. as previously described. Two days prior to challenge, and then every other day until completion of the experiment, 200µg anti-CD4 mAb (GK1.5; American Type Culture Collection, ATCC) and 200µg anti-CD8 mAb (TIB105; ATCC), or control mAbs (200µg SRF8-B6 and 200µg Y13-238)(23), were injected i.p. to the mice. At the end of the experiment, spleens were harvested for assessment of T-cell depletion. Briefly, single cell suspensions were prepared, and cells stained with FITC-conjugated anti-mouse
CD3e (35-0031, Tonbo Biosciences, San Diego, CA, USA), PerCP-Cy™5.5-conjugated rat anti-mouse CD45R (552771 BD Pharmingen), APC-conjugated rat anti-mouse CD4 (1540-11, Southern Biotech Associates, Birmingham, AL, USA), and PE-conjugated rat anti-mouse CD8a (553033, BD Pharmingen). For background assessments, the following isotype controls were used: APC-conjugated rat IgG2b (0118-11, Southern Biotech Associates, Birmingham, AL, USA) and PE-conjugated rat IgG2a (553930, BD Pharmingen). The data was analyzed with FlowJo 10.2 software. Representative flow panels of the stained splenocytes are shown in Fig. S3. The degree of depletion was >99%.

**Statistical analysis**

p-values represent exact values calculated by unpaired non-parametric two-tailed Mann-Whitney tests. Data treated with sigmoidal fitting software are represented with p values from the comparison by the extra sum-of-squares F test to determine significance of the IC levels. Correlation was computed using nonparametric Spearman correlation and represented with a two-tailed p value. Weight curves are analyzed with two-way ANOVA, and survival curves with the Gehan-Breslow-Wilcoxon test. All analysis was performed using GraphPad Prim 6 software.
Acknowledgments:

We thank Harvard Apparatus BTX for providing the skin electroporator, and Inovio Biomedical Co for providing the muscle electroporator. The technical help of Elisabeth Vikse is gratefully acknowledged. We thank the National Institute of Biological Sciences (NIBSC), UK for providing the influenza H7 strains.

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Competing interests:

BB and GG are inventors on patent applications filed on the vaccine molecules by the TTO offices of the University of Oslo and Oslo University Hospital, according to institutional rules. BB is head of the Scientific panel of Vaccibody AS, and holds shares in the company.

Author contributions:

TKA, FZ, RJC, BB, and GG conceived and designed experiments. TKA, FZ, and GG performed experiments. TKA, FZ, RJC, BB, and GG analysed the experiments. TKA, BB, and GG wrote the paper, but all authors contributed comments and editing.
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preferentially with α2,3-linked sialic acids and bind weakly to α2,6-linked sialic acids. J Gen Virol 94:2417-2423.


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Figure 1: Characterization of vaccine proteins. A) Schematic illustration of a dimeric vaccine protein. The targeting units, either a scFv specific for mouse I-E\textsuperscript{d} (αMHCII), the chemokine MIP1α (Mip1α), or a scFv specific for the hapten NIP (αNip) (non-targeted control), are connected to HA via a dimerization unit containing the hinge and C\textsubscript{H}3 domain from human IgG3. B) Western blot of supernatants from 293E cells transfected with the indicated vaccine plasmids. Molecular sizes and corresponding structures are indicated. C) Vaccine proteins in supernatants from transiently transfected 293E cells were detected in an ELISA specific for HA from A/Shanghai/1/2013 (H7N9). D) Binding of vaccine proteins to B-cells (CD19\textsuperscript{+}), macrophages (F4/80\textsuperscript{+} CD64\textsuperscript{+}), DCs (Lin\textsuperscript{-} CD11c\textsuperscript{hi}) divided into conventional DC 1 (CD24\textsuperscript{+}) and conventional DC 2 (CD11b\textsuperscript{+}), and T-cells (CD3\textsuperscript{+}) from BALB/c splenocytes.

Figure 2: MHCII-targeted HA vaccination with or without a multibasic cleavage site. A) Alignment of the MBCS in H7 and the deleted corresponding sequence in H7Δ. B) Schematic illustration depicting the vaccine monomer with indicated MBCS in HA. The full length vaccine monomer is ~150kDa and HA2 ~35kDa, resulting in fragments of ~115kDa and ~35kDa, respectively, under reducing conditions. C) Western Blot of secreted vaccine proteins (indicated) under reducing conditions in supernatant from transiently transfected 293E cells. D) Binding of secreted αMHCII-H7 or αMHCII-H7Δ proteins in supernatants from transiently transfected 293E cells detected in an ELISA specific for HA from A/Shanghai/1/2013 (H7N9). E) Mice (n=6/group) were vaccinated i.d. with plasmid DNA encoding either αMHCII-H7 or αMHCII-H7Δ, or NaCl, and IgG in sera measured in ELISA against recombinant HA
from influenza A/Shanghai/1/2013(H7N9) at weeks 1, 2 and 4 post vaccination.

*p<0.05, two-tailed Mann-Whitney test.

Figure 3: Antibody responses after a single DNA vaccination. A) Schematic illustration of the experiment. Briefly, BALB/c (n=12/group) or CB6F1 mice (n=6/group) were DNA vaccinated once i.d. with the indicated vaccine plasmids. Sera were collected up to 4 weeks post vaccination from BALB/c (B) and CB6F1 (C), and antibody responses measured in ELISA against recombinant HA from influenza A/Shanghai/1/2013(H7N9). Two-tailed Mann-Whitney test: *p<0.05 and **p<0.01.

D) Sera from BALB/c from week 2 and 4 were pooled by group, and assayed in a pseudotype microneutralization assay against A/FPV/Rostock/1934(H7N1). Neutralization curves were fitted with GraphPad Prism 6 software and IC<sub>50</sub> and IC<sub>90</sub> titers were calculated. Extra sum-of-squares F test: *p<0.05 and **p<0.01.

Figure 4. Induction of T cells after a single immunization. BALB/c mice (n=6/group, n=3/group for NaCl and H1 controls) were DNA vaccinated i.m. with plasmids encoding the indicated vaccines. Spleens were harvested at 8 weeks post vaccination, splenocytes re-stimulated with recombinant HA from A/Shanghai/1/2013(H7N9) (A), or HA from either A/Shanghai/1/2013(H7N9), or A/Vietnam/1194/2004(H5N1), or A/Puerto Rico/8/34(H1N1) (B), and the number of IFNγ-secreting cells evaluated. C) BALB/c mice (n=6/group) were vaccinated i.d. with plasmid DNA encoding the indicated vaccines. 5 weeks later, 5x10<sup>6</sup> A20 cells expressing cytosolic H7 and GFP, and 5x10<sup>6</sup> A20 cells expressing cytosolic mCherry were injected i.v. The prevalence of GFP or mCherry positive cells were assessed.
16h later, and killing ratios calculated. *p<0.05 and **p<0.01, two-tailed Mann-Whitney test.

**Figure 5. Viral challenge of DNA immunized mice.** BALB/c mice (n=6-12/group) or CB6F1 mice (n=6/group) were vaccinated with 25µg DNA i.d., and challenged with 20xLD50 of mouse adapted A/turkey/Italy/3889/1999 (H7N1) at week 5 post vaccination. Body weight (upper panels) was measured after challenge to assess morbidity, and survival curves (lower panels) are shown for mice receiving the indicated vaccines from A) BALB/c mice and B) CB6F1 mice. C) BALB/c mice were vaccinated with vaccines encoding the HA antigen A/turkey/Italy/3889/1999 (H7N1) homologous to the challenge strain and challenged at week 5 post vaccination. (Weight curves: *p<0.05 and **p<0.01, two way ANOVA. Survival curves: *p<0.05 and **p<0.01, Gehan-Breslow-Wilcoxon test). D) Representative micrographs of H&E stained sections of lungs from each group from the experiment in A) collected 7 days post challenge. Scale bar 250µm.

**Figure 6: Viral challenge of DNA immunized mice after depletion of T-cells.** Mice (n=8-10/group) were DNA vaccinated i.d. with the indicated vaccines, and then challenged with 20xLD50 A/turkey/Italy/3889/1999 (H7N1) 5 weeks post vaccination. Two days prior to challenge, and every other day until completion of the experiment, mice were injected i.p. with either CD4 and CD8 depleting mAbs, or isotype matched mAbs. Starting at the day of challenge (D0), weight was monitored in mice vaccinated with Mip1α-H7 (A), or αMHCII-H7 (B). *p<0.05 and **p<0.01, two way ANOVA. C) Survival curves for mice in A and B. *p<0.05 and **p<0.01, Gehan-Breslow-Wilcoxon test.
Figure 1

A. Targeting unit: αMHCII, Mip1α or αNip (non-targeted control)
Dimerization domain: hinge and CH3 from human IgG3
Antigenic unit: H7

B. 260kDa
140kDa
100kDa
70kDa
H7
αNip-H7
Mip1α-H7
αMHCII-H7
mock

C. 0
5
10
15
ng/ml
H7
αNip
Mip1α
αMHCII
mock

D. B-cells
MΦ
αMHCII-H7
Mip1α-H7
αNip-H7
iso.

Binding of vaccine protein
Figure 2

A  Antigen Sequence (MBCS in bold)

<table>
<thead>
<tr>
<th></th>
<th>H7</th>
<th>H7Δ</th>
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<tbody>
<tr>
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<td>334 E I P K G S R V R R G L F G A</td>
<td>334 E I P K G S - - - - G L F G A</td>
</tr>
</tbody>
</table>

B  

- αMHCII
- H7
- MBCS
- ~150kDa
- ~115kDa
- ~35kDa

C  

- 140kDa
- 100kDa
- 70kDa
- 50kDa
- 40kDa
- 35kDa

D  

- IgG titer (10^3)
- ng/ml

E  

- IgG titer (10^3)
- Weeks

- NaCl
- αMHCII-H7
- αMHCII-H7Δ
- mock

* indicates significance:

- * p < 0.05
- ** p < 0.01
- *** p < 0.001
Figure 3

A

NaCl or plasmid DNA:
- NaCl
- H7 25µg i.d.
- αNip-H7
- Mip1α-H7
- αMHCII-H7

BALB/c or CB6F1

Weeks

H7 PNA (d)
αH7 IgG (b-c)

B

IgG titer (x10^3)

Week

NaCl
H7
αNip-H7
Mip1α-H7
αMHCII-H7

C

IgG titer (x10^3)

Week

αMHCII-H7

D

IC_{50} titer (x10^3)

Week

αMHCII-H1
Figure 4

A

Stimulating agent vs Spots/10^6 cells

B

H7 H5 H1 mock

C

GFP mCherry

NaCl Mip1α-H7 αMHCII-H7

% Killing

**

10.3 93.8

48.2 51.8

6.25 89.7

0.045 0.048

0.045 0.048

NaCl Mip1α-H7 αMHCII-H7

**
Figure 5

A

B

C

D

NaCl

H7

αNip-H7

Mip1α-H7

αMHCII-H7

Days

% Body Weight

0 5 10 15

% Survival

0 5 10 15

% Body Weight

0 5 10 15

% Survival

0 5 10 15

% Body Weight

0 5 10 15

% Survival

0 5 10 15

% Body Weight

0 5 10 15

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% Body Weight

0 5 10 15

% Survival

0 5 10 15
Figure 6

(A) % Body Weight over Days for different treatments.

(B) % Body Weight over Days for different vaccines.

(C) % Survival over Days for different treatments.

<table>
<thead>
<tr>
<th>Vaccine</th>
<th>Treatment</th>
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<td>NaCl</td>
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<td></td>
</tr>
<tr>
<td>Mip1α-H7</td>
<td>T-cell depleted</td>
<td>●</td>
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<td></td>
<td>Isotype matched</td>
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